

In vitro phagocytosis of liquid-stored red blood cells requires serum and can be inhibited with fucoidan and dextran sulphate

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Vox Sanguinis

Background and Objectives Red-blood-cells (RBCs) undergo structural and metabolic changes with prolonged storage, which ultimately may decrease their survival after transfusion. Although the storage-induced damage to RBCs has been rather well described biochemically, little is known about the mechanisms underlying the recognition and rapid clearance of the damaged cells by macrophages.

Materials and Methods We, here, used a murine model for cold (+4°C) RBC storage and transfusion. Phagocytosis of human or murine RBCs, liquid stored for 6–8 weeks or 10–14 days, respectively, was investigated in murine peritoneal macrophages.

Results The effects of storage on murine RBCs resembled that described for stored human RBCs with regard to decreased adenosine triphosphate (ATP) levels, accumulation of microparticles (MPs) during storage, and RBC recovery kinetics after transfusion. Under serum-free conditions, phagocytosis of stored human or murine RBCs *in vitro* was reduced by 70–75%, as compared with that in the presence of heat-inactivated fetal calf serum (FCS). Human serum promoted phagocytosis of stored human RBCs similar to that seen with FCS. By adding fucoidan or dextran sulphate (blockers of scavenger receptors class A (SR-A)), phagocytosis of human or murine RBCs was reduced by more than 90%. Phagocytosis of stored human RBCs was also sensitive to inhibition by the phosphatidylinositol 3 kinase-inhibitor LY294002, the ERK1/2-inhibitor PD98059, or the p38 MAPK-inhibitor SB203580.

Conclusion RBCs damaged during liquid storage may be recognized by macrophage SR-A and serum-dependent mechanisms. This species-independent recognition mechanism may help to further understand the rapid clearance of stored RBCs shortly after transfusion.

Key words: blood processing, quality management, transfusion medicine (in general).

Received: 13 September 2019,
revised 3 March 2020,
accepted 26 March 2020,
published online 29 April 2020

Introduction

Transfusion of RBCs is a common practice in modern medical care, with roughly 90 million transfused units

each year worldwide [1]. RBCs evidently undergo metabolic and structural changes during liquid storage, which is referred to as the storage lesion [2]. Although the relationship to clinical outcome remains debated, the storage lesion may impair the function of RBCs and accelerate their short- and long-term clearance [3,4]. Prolonged storage may result in a rapid removal of as much as 25–30% of the transfused RBCs by the reticulo-endothelial

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system within 24 h, [5] likely causing a corresponding decrease in the anticipated clinical response of the recipient. In mice, rapid macrophage-mediated phagocytosis of stored RBCs has been linked to a profound pro-inflammatory cytokine storm due to iron overload [6,7]. So far, no clear evidence has been presented for such a response in humans, except for a report in preterm infants [8]. The mechanism behind this swift macrophage-mediated phagocytosis is not well understood, but is likely due to a combination of storage-related metabolic and structural changes within the RBC population. *In vitro* studies have indeed identified a number of time-dependent changes of liquid stored RBCs, including a reduction of intracellular ATP, increased oxidative stress, and membrane shape shifting, which could decrease the membrane deformability and increase vesiculation, where the latter results in accumulation of MPs in the storage medium [9,10]. *In vivo* aging of RBCs share several features to that described for the storage lesion; including reduced metabolic activity, oxidative damage and membrane loss through vesiculation [11].

The use of a murine model system has been shown to resemble several of the characteristics of human stored RBCs, regarding both the storage lesion and post-transfusion recovery [12] and could offer a possibility to study mechanistic aspects that are not possible to study in humans due to ethical considerations. It has been shown that experimentally oxidized RBCs can be phagocytosed by macrophages in a serum-dependent manner and that such uptake could be inhibited by blocking SR-A [13]. This raises the question whether this uptake mechanism could also be relevant for stored RBCs, since oxidative stress is one feature of the storage lesion [10].

Therefore, the aims of the present study were to characterize a model of murine RBC storage, which should resemble that for stored human RBCs, and to investigate the possibility that SR-A could be involved in macrophage recognition of stored RBCs.

Materials and methods

Mice

Adult male or female C57BL/6 mice were used in the experiments. Mice were kept under low pathogen conditions and bred in our local facility with free access to water and food in accordance with local guidelines. All the experiments were performed in compliance with relevant Swedish and institutional laws and guidelines and approved by the Umeå research animal ethics committee (A14-12).

Preparation and storage of human RBC

Human RBCs were acquired from blood bags (containing saline-adenine-glucose-mannitol (SAG-M)) kept at a constant temperature of +4°C and stored in the normal deposit at the Blood Bank at the University Hospital of Umeå (Umeå, Sweden). A part of a plastic tube, filled with ~1 ml RBCs, was welded off from blood bags stored for up to six weeks. The acquired samples were anonymized and carried only information of blood group and the date of donation. Since the results obtained in the present study, could not be traced back to a specific blood donor, ethical approval was not needed according to Swedish legislation (41, SFS 2003:460).

Preparation and storage of mouse RBC

Whole blood was collected in citrate-phosphate-dextrose (CPD, blood to CPD-ratio - 7:1) by heart puncture of euthanized mice and spun at +4°C and 500x *g* for 5 min. Plasma was then transferred to a separate tube, while the buffy coat and the top layer of the RBCs were discarded. Plasma was spun at +4°C and 1300x *g* for 10 min, in order to remove any residual cells, after which the supernatant was transferred into a new tube. RBCs were washed 3 times in PBS and the top layer of RBC containing any residual white-blood-cells and platelets were removed after each wash. SAG-M and plasma (ratio 3:1) were then added to the washed RBCs to a haematocrit (Hct) of ~40% and transferred to 0.5 ml tubes and either used fresh (<24 h) or left for storage 10–14 days at + 4°C.

ATP measurement

Intracellular ATP levels were measured by lysing 5×10^7 cells in cold dH₂O (ratio 1:100) followed by incubation on ice for 15 min. ATP samples of known concentrations were prepared in the range of 78 to 364 nM to generate a standard curve. Each sample (in triplicate) was mixed with ATP reagent (50 mM HEPES, 20 mM KCl, 5 mM MgCl₂, 0.5 mg/ml albumin, 8.5 mM luciferin and 1 μM luciferase in dH₂O, pH 7.6) at a 1:10 ratio, incubated on ice for 15 min and finally measured for peak luminescence in a Triathler luminometer from Hidex (Turku, Finland). RBC ATP levels were calculated from the ATP standard curve and expressed as % of day 0 values for all time points.

Microparticle quantification

Freshly isolated or 10 days stored murine RBC concentrates, prepared as described above, were incubated with

fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ter-119 (ImmunoTools, Friesoythe, Germany), FITC-conjugated anti-mouse CD45 (ImmunoTools) or phycoerythrin (PE)-conjugated anti-mouse CD61 (PharMingen San Diego, CA) on ice for 30 min. Samples were then analysed for Ter-119⁺CD45⁻CD61⁻ MPs using logarithmic scaling for forward and side scatter on a FACSCalibur flow cytometer (BD Biosciences, Mountain view, CA) and results were analysed by CellQuest software (BD Biosciences).

RBC clearance experiments

In vivo biotinylation of RBCs was carried out essentially as previously described, [14] with some modifications. In brief, mice were injected intravenously with 1.1 mg of N-hydroxysuccinimidobiotin (EZ-Link NHS-Biotin, Thermo Scientific, Rockford, IL) in 150 µl PBS via a lateral tail vein. The next day, whole blood was collected by cardiac puncture in euthanized mice, after which RBCs were isolated and stored as described above. For transfusions, 150 µl of freshly isolated or 10 days stored RBCs were injected via a lateral tail vein into recipient mice, anaesthetized with isoflurane (Baxter, IL, USA). Five microlitres of whole blood were then taken from the contralateral tail vein at indicated time points after transfusion and resuspended in 100 µL of PBS containing 5 mM EDTA. Samples were washed in PBS and incubated with streptavidin-PE (Nordic Biosite, Täby, Sweden) for 25 min on ice. Cells were then washed and analysed on a FACSCalibur flow cytometer (BD Biosciences, Mountain view, CA) and the CellQuest software (BD Biosciences).

Phagocytosis experiments

Phagocytosis experiments were performed as described elsewhere, [13] with some modifications. In brief, macrophages were harvested by flushing the peritoneal cavity of euthanized mice with DMEM media containing 10 % FCS. Cells were then sedimented by centrifugation and resuspended to 1×10^6 cells/mL in DMEM + 10 % FCS. The cell suspension (300 µl) was then added to 11 mm sterile glass coverslips in 4 well plates and incubated at 37°C, 5% CO₂ for 2 h. Non-adherent cells were then washed off using warm (37°C) PBS, leaving only adherent macrophages on the glass coverslips. New DMEM medium, with or without 10% FCS, 10% human serum (HS), 100 µg/ml fucoidan, dextran sulphate or chondroitin sulphate was then added to the coverslips. For signalling inhibitor experiments, macrophages were preincubated for 30 min with DMEM/ 10% FCS and the p38 MAPK-inhibitor SB203580 (30 µM), the ERK1/2-inhibitor PD98059 (30 µM) or the phosphatidylinositol 3 (PI3) kinase-

inhibitor LY294002 (50 µM). Fifty microlitres of RBCs, diluted to 3% Hct, was then added followed by incubation for 1 h at 37°C, 5% CO₂. After washing in cold PBS, non-ingested RBCs were lysed by adding dH₂O for 30 s, followed by fixation and staining by May-Grünwald/Giemsa. At least two coverslips per condition were used in every experiment. A phagocytosis index was calculated by counting the number of phagocytosed RBCs in at least 300 macrophages/coverslip and expressed as [number of phagocytosed targets/total number of macrophages] x 100.

Statistics

Statistical analyses were performed using Student's *t*-test for paired or unpaired comparisons, as described in the figure legends.

Results

Characteristics of stored murine RBCs

To investigate if the effects of cold storage on murine RBCs resembled that reported for cold stored human RBCs, we first measured the intracellular ATP concentration during storage for up to 10 days. The RBC ATP content in the stored murine RBC units was not significantly reduced after 4 days of storage (Fig. 1a). However, after 7 and 10 days the ATP content was reduced to $69.1 \pm 2.9\%$ and $47.2 \pm 1.9\%$, respectively, of that seen in freshly isolated RBCs (Fig. 1a). Formation of RBC MPs has been recognized as an important feature during storage of human RBCs [15]. Therefore, we next investigated if storage of murine RBCs resulted in the formation of MPs. This analysis was based on flow cytometric analysis to identify smaller cell fragments accumulating during RBC storage (Fig. 1b,c) [9]. While freshly isolated RBC preparations only contained $0.45 \pm 0.04\%$ MPs, the amount of MPs was increased to $19.1 \pm 3.8\%$ of all cellular events following 10 days of storage (Fig. 1b-d). The vast majority of these MPs expressed the erythroid cell marker Ter-119, but lacked expression of the leucocyte antigen CD45 (Fig. 1e) and the platelet antigen CD61 (data not shown), indicating that they were originating from RBCs. Based on the expression of CD61, we also quantified the amount of platelets in the RBC preparations before storage and found that $0.07 \pm 0.02\%$ of all cells were platelets. In humans, long-term RBC storage (>35 days) may lead to the disappearance of 25% or more of the transfused RBCs within 24 h [5]. We, therefore, next investigated the *in vivo* clearance of transfused murine RBCs stored for 10 days. Fresh RBCs showed virtually no clearance from the circulation within the first 24 h after transfusion

(Fig. 1f). In marked contrast, $36.4 \pm 2.5\%$ of the stored RBCs disappeared from the circulation within 24 h (Fig. 1f). It is generally assumed that the fraction of transfused stored RBCs remaining in circulation after the first 24 h should have a close to normal survival time [16]. Indeed, we did also find that the clearance of fresh and stored murine RBCs was virtually similar when comparing the relative clearance-rate of RBCs that remained in circulation at 24 h after transfusion (Fig. 1g). Thus, the storage model for murine RBCs seemed to match many of the characteristics described for human RBCs during long-term storage at $+4^{\circ}\text{C}$.

Serum-dependent phagocytosis of stored human or murine RBCs in murine resident peritoneal macrophages

Next, we wanted to start exploring the hypothesis that evolutionary conserved recognition mechanisms could be involved in the phagocytosis of RBCs that have been damaged during storage. Such recognition mechanisms are generally not species-dependent, [17–19] which would allow us to use mouse peritoneal macrophages in phagocytosis assays with stored human or murine RBCs as targets. That human RBCs were not recognized as ‘foreign’, *per se*, was shown by the complete absence of phagocytosis when freshly isolated human or murine RBCs were incubated with the macrophages (data not shown). In marked contrast, we found substantial phagocytosis when stored human (Fig. 2a) or murine RBCs (Fig. 2b) were used as targets. Since it has been shown that phagocytosis of oxidatively damaged RBCs is serum-dependent, [13,20], we investigated if the same applied for stored human or murine RBCs. Under serum-free conditions, we found that phagocytosis of stored human or murine RBCs was reduced by 70–75%, as compared with that in the presence of heat-inactivated FCS (Fig. 2a,b). Importantly, heat-inactivated human serum conferred the same stimulatory effect on phagocytosis of stored human RBCs, as that observed in the presence of FCS (Fig. 2a).

The role of scavenger receptors in phagocytosis of stored RBCs

The finding that SR-A could mediate uptake of oxidized murine RBCs, [13] led us to investigate if scavenger receptors could also be involved in the phagocytosis of stored RBCs. For this, we used fucoidan or dextran sulphate, compounds known to inhibit SR-A [21]. In the presence of fucoidan, phagocytosis of stored human RBCs was only $8.9 \pm 1.7\%$ of that seen in the control (Fig. 3a) and phagocytosis of stored murine RBCs was $7.8 \pm 3.0\%$ of that in the absence of fucoidan (Fig. 3b). Dextran

sulphate showed an even stronger inhibitory effect and virtually abolished phagocytosis of stored human or murine RBCs, whereas the structurally similar negative control chondroitin sulphate did not significantly affect phagocytosis (Fig. 3a,b).

Signalling involved in macrophage phagocytosis of stored RBCs

We have previously shown that both $\text{Fc}\gamma$ receptor-mediated uptake of IgG-opsonized RBCs and scavenger receptor-mediated uptake of oxidized RBCs were sensitive to inhibition of PI3 kinase, p38MAPK or ERK1/2 [13]. Therefore, we further investigated if these intracellular signalling pathways were also involved in regulating phagocytosis of stored RBCs. These experiments revealed that phagocytosis of stored human RBCs was reduced by >90% in the presence of the PI3 kinase-inhibitor LY294002 (Fig. 4). In addition, the p38MAPK-inhibitor SB203580 and the ERK1/2-inhibitor PD98059 each reduced phagocytosis of stored RBCs by 36% and 30%, respectively (Fig. 4).

Discussion

In the present study, we show that stored RBCs can be recognized and phagocytosed by macrophages via a mechanism that is species-independent, dependent on serum, and likely involves SR-A. The well-known fact that a substantial amount of stored RBCs may rapidly disappear within 24 h after transfusion has so far not been met by a thorough analysis of the mechanisms mediating this elimination. Despite the many differences between nucleated cells and RBCs, it is still plausible to hypothesize that macrophage recognition and uptake of RBCs damaged during storage could at least in part be mediated by mechanisms similar to that described for the uptake of apoptotic cells. Phagocytosis of apoptotic cells has been associated with a profound anti-inflammatory effect, while rapid clearance of stored RBCs has been associated with pro-inflammatory responses, at least in experimental systems [6]. Therefore, a more detailed understanding of the recognition and uptake mechanisms involved in the rapid clearance of transfused RBCs may help to understand what immune/inflammatory responses could result from this RBC uptake.

The first objective of the present study was to confirm an experimental model of stored murine RBCs, which could be used in parallel with analyses of stored human RBCs. An important reason to establish murine models is the ability to follow clearance *in vivo* in more detail to pinpoint specific mechanisms [22]. However, a direct comparison is not straightforward for several reasons; for

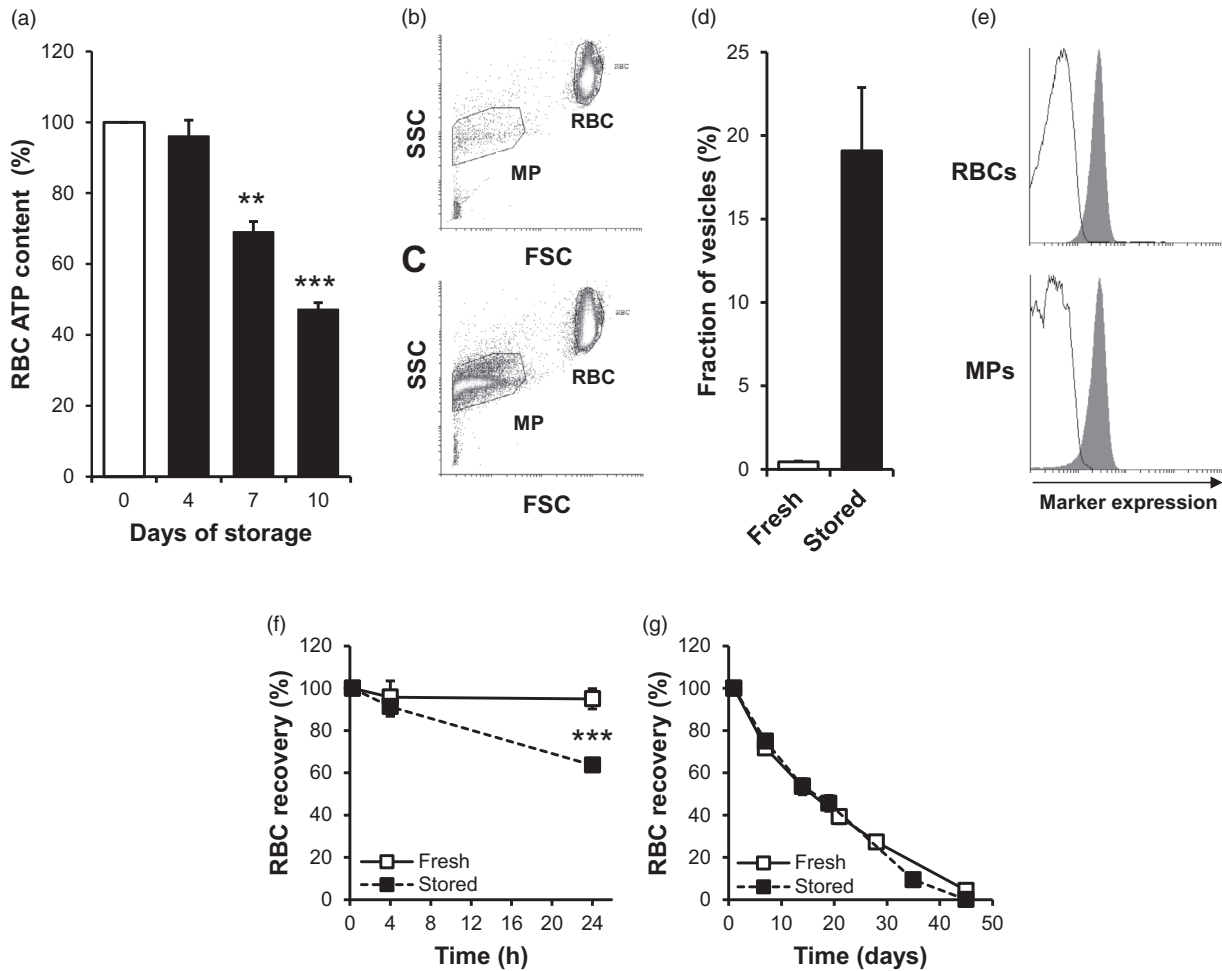


Fig. 1 Characteristics of stored murine RBCs. (a) Intracellular ATP content at 0, 4, 7 and 10 days of storage in murine RBCs kept at +4 °C in SAG-M and autologous plasma (ratio 3:1) at a Hct of 40%. The ATP content is expressed as per cent of that at day 0. Data are mean \pm SD of three individual experiments. ** $P < 0.01$ and *** $P < 0.001$, as compared with day 0 using Student's *t*-test for paired analyses. (b, c) The presence of MPs was evaluated by flow cytometry. Forward scatter (FSC) and side scatter (SSC) plots of RBCs in storage media from freshly isolated (b) or 10 day stored (c) RBCs showed the accumulation of MPs at day 10 of storage. Shown are representative data from 4 (fresh RBCs) or 6 (stored RBCs) individual samples. (d) The fraction of MPs was expressed in per cent of total events in freshly isolated RBCs (open bar) or 10 days stored RBCs (black bar). Data are mean \pm SD of 4 (fresh RBCs) or 6 (stored RBCs) individual samples. (e) Both the RBC and MP populations expressed the erythrocyte marker TER-119 (grey histogram) but not the leucocyte marker CD45 (open histogram). Shown are representative data from 4 (fresh RBCs) or 6 (stored RBCs) individual samples. (f) Twenty four hour recovery of fresh (open squares) or 10 days stored RBCs (black squares). Data are mean \pm SD of 4 mice per group. *** $P < 0.001$, using Student's *t*-test for unpaired analysis. (g) Long-term survival of transfused fresh (open squares) or 10 days stored (black squares) RBCs when the amount of RBCs remaining in circulation at 24 h was set to 100%. Data are mean \pm SD of 4 mice per group.

example, the average RBC life-span is 120 days in humans and 50 days in mice, [23,24] and murine RBCs are generally more fragile than human RBCs. This could explain why storage of murine RBCs for 10–14 days results in changes similar to that seen in human RBCs stored for 6 weeks. Our present data indicated that the storage conditions we used for murine RBCs indeed showed several important similarities with that observed for stored human RBCs. First, since it has been well established that the ATP content of stored human RBCs is

reduced over time during storage, and that this reduction correlates with the 24 h *in vivo* recovery, [25] it was interesting to note that the kinetics of ATP loss observed in stored murine RBCs seemed to follow what should be expected based on previous findings in human RBCs stored in CPD-SAG-M [5,9]. Second, we found formation of storage-induced murine RBC-derived MPs, corresponding well to the reported formation of MPs during storage of human RBCs [9,10]. Third, upon transfusion of stored murine RBCs, we found that about 35% of the cells

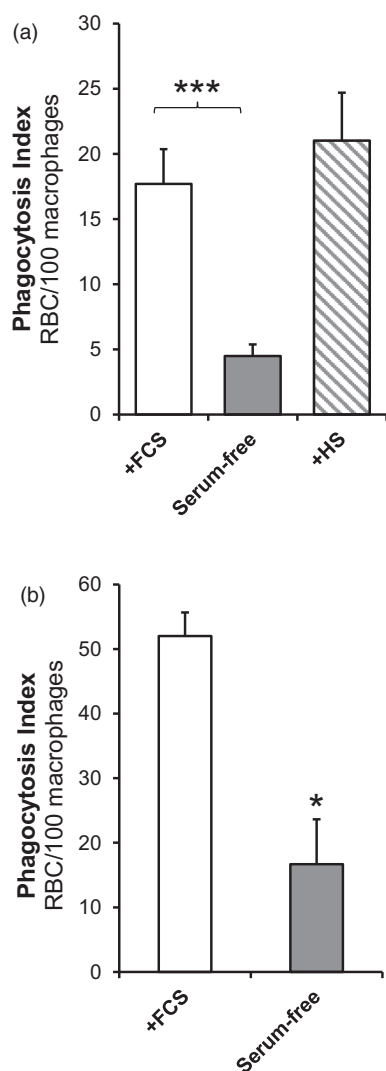


Fig. 2 *In vitro* macrophage phagocytosis of stored human or murine RBCs is serum-dependent. (a) Phagocytosis of human RBCs stored for 6 weeks or (b) murine RBCs stored for 10–14 days after 60 min incubation with mouse peritoneal macrophages in the presence of 10 % FCS (open bars), 10 % human serum (hashed bar) or in serum-free media (grey bars). Data are mean \pm SD of three separate experiments. * $P < 0.05$ and *** $P < 0.001$, using Student's *t*-test for paired analyses.

disappeared from the circulation within 24 h, while the RBCs surviving past that time-point showed virtually normal survival in circulation. The latter observation also fits well with what has been described for stored human RBCs [5]. Taken together, these findings support that the stored murine RBCs used in the present study fulfils key requirements to be compared with stored human RBCs.

Serum may contain several factors which can function as opsonins or bridging molecules to facilitate the interaction between a specific molecule/structure on a target cell and a tethering or pro-phagocytic receptor on a

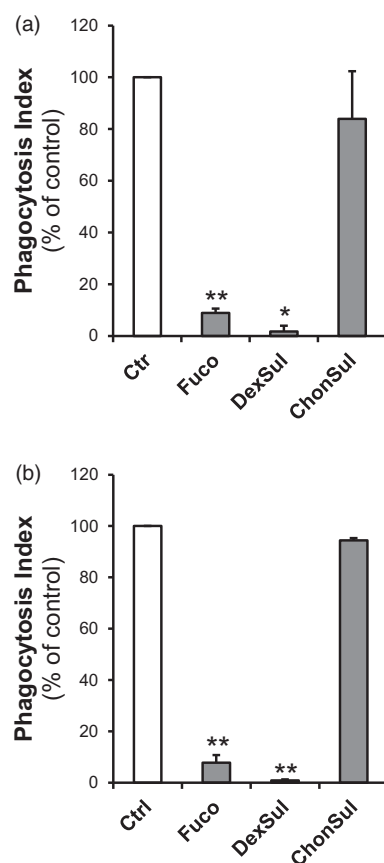


Fig. 3 *In vitro* phagocytosis of stored RBCs is sensitive to inhibitors of class A scavenger receptors. (a) Phagocytosis of human RBCs stored for 6 weeks, or (b) murine RBCs stored for 10–14 days, after 60 min incubation with mouse peritoneal macrophages in medium containing 10 % FCS (Ctr), and following addition of 100 μ g/ml fucoidan (Fuco), 100 μ g/ml dextran sulphate (DexSul) or 100 μ g/ml chondroitin sulphate (ChonSul). Data are mean \pm SD of 3–5 individual experiments. * $P < 0.05$ and ** $P < 0.01$, as compared with that in the absence of inhibitor, using Student's *t*-test for paired analyses.

phagocyte [20,26]. We indeed found that phagocytosis of stored human or murine RBCs was reduced by 70–75% in the absence of serum. This effect seemed to be mediated by a heat-stable (56°C) serum factor and did not seem to be species-specific, since FCS and human serum promoted phagocytosis of human RBCs in the same manner. It has been suggested that naturally senescent RBCs in circulation, as well as stored RBCs, accumulate degraded band 3 proteins, which can bind naturally occurring antibodies [11]. When the amount of such antibodies reaches a certain threshold, it could induce Fc γ receptor-mediated phagocytosis of senescent or damaged RBCs. In the present study, we did not specifically investigate the contribution of Fc γ -receptors in mediating the phagocytosis of stored RBCs. However, we found that fucoidan and dextran sulphate strongly inhibited the phagocytosis of stored human

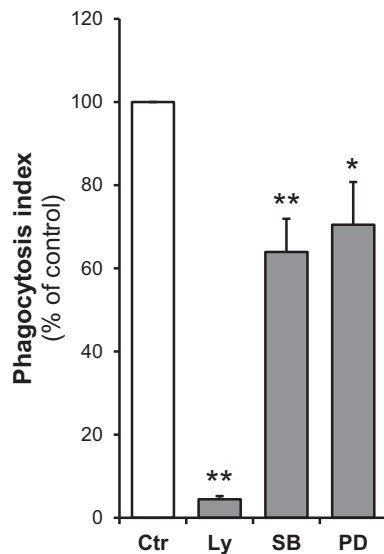


Fig. 4 *In vitro* phagocytosis of stored RBCs is regulated by PI3 kinase, p38MAPK and ERK1/2. Phagocytosis of human RBCs stored for 6 weeks after 60 min incubation with mouse peritoneal macrophages in medium containing 10 % FCS. Macrophages were preincubated in the absence of inhibitor (Ctr), or in the presence of the PI3 kinase-inhibitor LY294002 (50 μ M; Ly), the p38 MAPK-inhibitor SB203580 (30 μ M; SB) or the ERK1/2-inhibitor PD98059 (30 μ M; PB). Data are mean \pm SEM of 5 individual experiments. * P < 0.05 and ** P < 0.01, as compared with that in the absence of inhibitor, using Student's *t*-test for paired analyses.

as well as murine RBCs in the presence of serum. Both fucoidan and dextran sulphate have been well established to inhibit SR-A and blocks the binding of other ligands to this particular group of receptors [13,21,27].

Depending on the colocalization of SR-A with other signalling and/or transport proteins, there are multiple

parallel intracellular signalling pathways that could theoretically be induced as a result of SR-A activation [28]. PI3 kinase, p38MAPK and ERK1/2 are part of these signalling pathways, [29-31] and the inhibitory effects from blocking these pathways, found by us and others, [7,32] therefore further supports SR-A dependent phagocytosis of stored RBCs. The findings do not however preclude a role of other receptor-mediated pathways.

In conclusion, the present study contributes to further understanding how RBCs that have been damaged during cold storage can be recognized by macrophages. While we provide novel findings of a possible involvement of SR-A and serum factors, a more detailed understanding of these interactions requires further investigation. The ability to use stored murine RBCs, with characteristics similar to that of stored human RBCs, will enhance the possibilities to understand the details of this recognition mechanism both *in vivo* and *in vitro*.

Acknowledgements

We thank Ms. Barbro Borgström for excellent technical assistance.

Funding

Supported by grants from the Swedish Research Council (2012-2702; PAO) and the Faculty of Medicine Foundations at Umeå University.

Conflict of interests

The authors have no conflicts of interest.

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