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Dose-dependent inhibitory effect of CD47 in macrophage uptake of IgG-opsonized murine erythrocytes ☆

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Abstract

The cell surface glycoprotein CD47 on target cells can bind to the inhibitory receptor SIRPα on macrophages to inhibit phagocytosis of antibody sensitized blood cells. The aim of this study was to determine if CD47 dose-dependently can regulate macrophage uptake of IgG-opsonized RBCs. $CD47^{+/-}$ RBCs express about 50% of the CD47 level found on $CD47^{+/+}$ RBCs. When injected into $CD47^{+/+}$ mice, $CD47^{+/-}$ RBCs showed a significantly faster antibody-mediated clearance as compared with $CD47^{+/+}$ RBCs injected into the same recipient. *In vitro* phagocytosis experiments confirmed that $CD47^{+/-}$ RBCs were taken up significantly more than $CD47^{+/+}$ RBCs, but significantly less than $CD47^{-/-}$ RBCs. A reduction in RBC CD47 expression just below 50% of that in normal RBCs can significantly accelerate RBC clearance by macrophages in the presence of RBC autoantibodies. This may have relevance for transfusion of stored RBCs, where loss of CD47 is seen over time, and in clearance of these cells by antibody-dependent phagocytosis.

Keywords: Red blood cell; Phagocytosis; SIRPα; Fc receptor

At the end of the normal RBC life-span, or in autoimmune hemolytic anemia (AIHA) where RBCs are opsonized by RBC-specific autoantibodies [1], these cells are recognized and phagocytosed by macrophages. The exact mechanisms involved in clearance of senescent RBCs are not completely understood. However, it has been proposed that a combination of changes in plasma membrane components, biomechanical changes, and natural antibodies are involved in mediating clearance of senescent RBCs [16]. The ubiquitously expressed cell surface glycoprotein CD47 (integrin-associated protein/IAP) is highly expressed by RBCs and can function as a marker of self to inhibit macrophage phagocytosis of IgG or complement-opsonized RBCs [2]. CD47 is a ligand for signal regulatory protein alpha (SIRPα/SHPS-1/BIT/MFR/P84) [3], a cell surface receptor of the immunoglobulin (Ig)-superfamily [4]. Although ubiquitously expressed, SIRPα is highly expressed on myeloid cells, such as monocytes, macrophages, dendritic cells and granulocytes [4–6]. The cytoplasmic domain of SIRPα contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [4]. Following CD47/SIRPα interaction, these ITIMs are phosphorylated and function as sites for binding the Src-homology 2 (SH2)-domain-containing tyrosine phosphatase SHP-1, which is needed to mediate inhibition of phagocytosis [2]. Absence of CD47 makes IgG-opsonized RBCs from CD47-deficient ($CD47^{-/-}$) mice very sensitive to macrophage phagocytosis, as compared with that for equally opsonized $CD47^{+/+}$ RBCs [2], and $CD47^{-/-}$ mice are severely sensitive to experimental AIHA [7]. We have recently shown that the same is true also for IgG-opsonized platelets, making $CD47^{-/-}$ mice more sensitive to experimental ITP [8]. Despite a 40–50% reduction in CD47 expression levels, $CD47^{+/-}$ mice were not found to be more sensitive in experimental models of AIHA (Oldenborg, et al., unpublished observations) or ITP [8], as compared with that seen in $CD47^{+/+}$ mice. However, *in vitro* platelet

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phagocytosis experiments using IgG-opsonized platelets and $CD47^{+/+}$ macrophages revealed that $CD47^{+/-}$ platelets were phagocytosed significantly more than equally opsonized $CD47^{+/+}$ platelets, but significantly less than equally opsonized $CD47^{-/-}$ platelets [8]. This suggests that there is a dose-dependent effect of CD47 in regulating Fcγ receptor-mediated phagocytosis of platelets, and that partial or absolute absence of CD47 in mice (i.e. $CD47^{+/-}$ or $CD47^{-/-}$ mice) could result in various levels of compensation, although the mechanisms behind this phenomenon are not yet understood.

The red cell storage lesion involves biochemical and biomechanical changes to RBCs during storage. Changes to the CD47 expression levels of stored RBCs have been reported, but with conflicting results. Anniss and Sparrow reported a small but significant (less than 10%) loss of CD47 during storage of RBCs for 28–42 days, where soluble CD47 could be detected in the storage medium [9]. However, another study showed a 52% loss of CD47 by day 24 of RBC storage [10]. Thus, despite the remarkable difference between these two reports, it is reasonable to expect that the red cell storage lesion involves loss of CD47.

It is generally thought that clearance of senescent RBCs from the circulation is a multifactorial process, where opsonization by natural antibodies may be an important step [16]. Thus, as a result of our previous findings on a dose-dependent effect of CD47 in regulating Fcγ receptor-mediated platelet clearance, and the fact that CD47 may be lost from RBCs during storage, the aim of the present study was to investigate if CD47 could dose-dependently regulate Fcγ receptor-mediated phagocytosis of RBCs.

Materials and methods

Reagents. The mouse anti-mouse RBC mAb 34-3C (IgG2a), established from autoimmune-prone NZB mice [11], and rat anti-mouse CD47 mAb miap301 [12] were purified from hybridoma supernatants by ammonium sulfate precipitation and protein-A chromatography (Amersham Biosciences, Piscataway, NJ). Rabbit antiserum against murine RBC was from Cappel, and the rabbit anti-murine RBC IgG fraction was purified by protein-G chromatography (Amersham Biosciences, Piscataway, NJ). DPX mounting medium was from VWR, Stockholm, Sweden. PKH 26 and 67, secondary antibodies and other reagents were from Sigma–Aldrich, St. Louis, MO, USA.

Mice. Male and female $CD47^{-/-}$ Balb/c mice [12], backcrossed to Balb/c (Jackson Laboratory, Bar Harbor, ME) for 16 generations, and their heterozygous and homozygous littermates were from our own breeding colony. Animals were kept in accordance with local guidelines and maintained in a specific pathogen-free barrier facility. All animal procedures were approved by Institutional Review Committees.

Flow cytometric analysis of RBC CD47 expression. Blood was obtained through a tail vein and immediately diluted in PBS/5mM EDTA. The blood was then washed in PBS and incubated with anti-CD47 mAb miap301 for 20 min on ice, followed by another wash with PBS. RBCs were then incubated with a secondary FITC-conjugated goat anti-rat IgG for 20 min, washed, and analysed using a FACscan flow cytometry (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences).

PKH 26 and PKH 67 staining. RBCs were stained with PKH 26 or PKH 67 according to the manufacturers instructions. In brief, blood was obtained by cardiac puncture of euthanized mice, using heparin as anticoagulant. The blood was then washed two times in PBS. After the last wash the RBCs were resuspended in diluent C (5×10^8 RBC/ml) and incubated with 4 μM red fluorescent dye PKH 26 or green fluorescent dye PKH 67 for 5 min at room temperature followed by addition of equivalent volume of PBS/HSA 2% for 1 min. The blood was then washed two times with PBS and resuspended in sterile 0.9% saline to a concentration of 3×10^9 RBC(s)/ml.

RBC survival experiments. PKH stained $CD47^{+/+}$ and $CD47^{+/-}$ RBCs were combined at a 1:1 ratio, were PKH-67 stained $CD47^{+/+}$ cells were mixed with PKH-26 stained $CD47^{+/-}$ RBCs, or the other way around to exclude the possibility that the specific labelling could affect the results. We obtained identical results, irrespective of the labelling used for RBCs of either genotype (data not shown). Recipient mice were anesthetized using isoflurane, followed by an intravenous (i.v) injection in a lateral tail vein with 350 μl of the RBC mixture at a concentration of 3×10^9 RBCs/ml, which results in approximately 2% fluorescent RBCs of respective phenotype. Twenty four hours after the RBC injection, the mice were injected intraperitoneally (i.p) with the anti-RBC antibody 34-3C at a concentration of 1 or 3 μg/g body weight, as previously described [7]. Clearance of injected RBCs was then monitored daily for 1 week in blood samples, using flow cytometry [7]. For this, blood (4 μl) was collected from the tail vein and immediately diluted in 600 μl PBS/5 mM EDTA, and further diluted five times in PBS before analysis. The mean value of the fraction of fluorescent RBCs at 60 min and 24 h after the RBC injection, in relation to the total number of RBCs in recipient blood, was used as a reference and set to 100%.

Measurement of hematocrit. For determination of Hct, blood (7 μl) was obtained from a tail vein and was immediately diluted in 95 μl cold PBS/5 mM EDTA. The blood was then kept on ice until further analysis (which was within 30 min). Hct was then determined using an automatic blood analyser (Sysmex KX-21; Sysmex Europe GMBH, Norderstedt, Germany). Before analysis, the blood samples were diluted in 200 μl diluting reagent (Cellpack; Sysmex) to give a final dilution of 2.3/100. With this dilution of mouse blood the measured Hct values are within the blood analyzers normal range. To confirm the accuracy of Hct measurement on the Sysmex blood analyzer, experiments were also performed were blood (70 μl) was collected into heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA), from the retroorbital vein plexa of anesthetized mice. The hematocrit tubes were spun in a hematocrit centrifuge (ICN, Needham Heights, MA), and the hematocrit was determined as the relative height of the RBC column expressed in percent. The two methods were found to give virtually similar results (data not shown).

Preparation of bone marrow-derived macrophages. Murine bone marrow-derived macrophages were prepared as previously described [8,13]. In brief, the bone marrow was flushed out of femoral bones with PBS/1% HSA. Following lysis of red blood cells, the bone marrow cells were resuspended in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and streptomycin (DMEM/10% FCS), plated on tissue-culture dishes, and incubated for 2 h at 37°C and 5% CO_2. Thereafter, non-adherent cells were resuspended in DMEM/10% FCS supplemented with 15% L929 cell supernatant (as a source of macrophage colony-stimulating factor [M-CSF]) and plated on bacterial plastic dishes for 6 days. Mature macrophages (5×10^4/glass) were then plated on glass coverslips in 4-well plates, and cultured in DMEM/10% FCS without M-CSF for 24 h before used in phagocytosis experiments.

In vitro phagocytosis experiments. RBCs were obtained by cardiac puncture, as described above, and washed two times in PBS followed by opsonization for 20 min at 37 °C with 200 μg/ml rabbit anti-murine RBC IgG at a concentration of 5×10^8 RBCs/ml. The cells were then washed two times in PBS. To compare the level of opsonization, samples of opsonized RBCs were incubated with Alexa488-conjugated goat anti-rabbit IgG and analysed by flow cytometry. After the last wash the RBCs were resuspended in DMEM/FCS 10% and added to the macrophages at 1×10^7/well. RBCs were allowed to adhere to the macrophages on ice for 20 min, followed by incubation for 30 min at 37°C and 5% CO_2. Following lysis of uningested RBCs with distilled water for 40 s, the macrophages were fixed and stained with May-Grünewald/Giemsa. The coverslips were then washed with distilled water, allowed to air dry for at least 30 min, and

mounted in DPX mounting medium. Ingested RBCs were counted using light microscopy, and a phagocytic index was calculated as the number of ingested RBCs/100 macrophages.

Statistics. Statistical analysis was performed by using the two-tailed Student's *t*-test for paired samples. All results are expressed as means ± SEM.

Results

CD47 expression levels on murine CD47$^{+/+}$ and CD47$^{+/-}$ RBC

We first compared the CD47 expression levels on CD47$^{+/-}$ and CD47$^{+/+}$ RBCs, using flow cytometry. These experiments revealed that the amount of CD47 expressed on CD47$^{+/-}$ RBCs was 46.5 ± 1.0% of that expressed by CD47$^{+/+}$ RBCs (Fig. 1).

RBC survival in vivo in the absence or presence of anti-RBC antibodies

Next, we determined the clearance rate of CD47$^{+/+}$ and CD47$^{+/-}$ RBCs in CD47$^{+/+}$ recipients in the absence of anti-RBC antibodies. For this, fluorescently labelled CD47$^{+/+}$ and CD47$^{+/-}$ RBCs (1:1 mixture), labelled with red or green dyes respectively, were injected intravenously. These experiments revealed that unopsonized CD47$^{+/-}$ RBCs were not cleared faster than unopsonized CD47$^{+/+}$ RBCs during the 7 day period of analysis (Fig. 2). To study the physiological consequences of a reduced CD47 expression on antibody-mediated RBC clearance *in vivo*, CD47$^{+/+}$ mice were injected with a 1:1 mixture of fluorescently labelled CD47$^{+/+}$ and CD47$^{+/-}$ RBCs, as described above. After that, the anti-RBC antibody 34-3C was injected at 3 or 1 μg/g body weight, and clearance of injected RBCs and Hct were followed every 24 h. In experiments with the higher dose of the 34-3C antibody (3 μg/g), the Hct dropped gradually reaching a nadir at day 4 after injection, at which time the Hct was reduced by 44% as compared with that on day 0 (Fig. 3A). During this time, clearance of CD47$^{+/-}$ RBCs was significantly more rapid than that seen for CD47$^{+/+}$ RBCs (Fig. 3B). Using the lower dose of the 34-3C antibody (1 μg/g), the Hct was reduced by 22% on day 4, as compared with that at day 0 (Fig. 3C). However, also here CD47$^{+/-}$ RBCs were cleared significantly more rapid than CD47$^{+/+}$ RBCs, and the difference was even more pronounced than that seen with the antibody at 3 μg/g body weight (Fig. 3D).

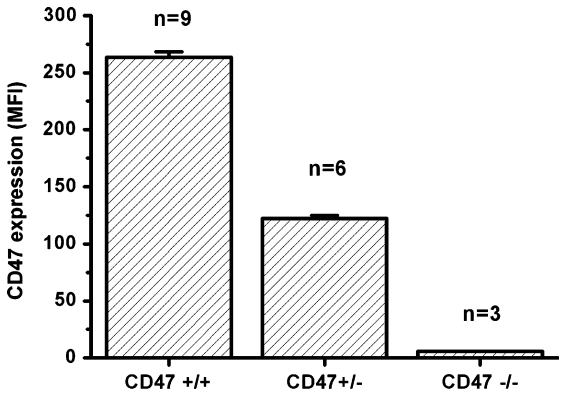

Fig. 1. Expression levels of CD47 in CD47$^{+/+}$, CD47$^{+/-}$, or CD47$^{-/-}$ RBCs. RBCs from CD47$^{+/+}$, CD47$^{+/-}$, or CD47$^{-/-}$ mice were labelled with anti-CD47 mAb miap301, followed by FITC-conjugated secondary antibodies, and analyzed by flow cytometry. Data are expressed as mean fluorescence intensity (MFI) for the number of mice indicated. The CD47 expression level in CD47$^{+/-}$ RBCs was 46.5 ± 1.0% of that in CD47$^{+/+}$ RBCs.

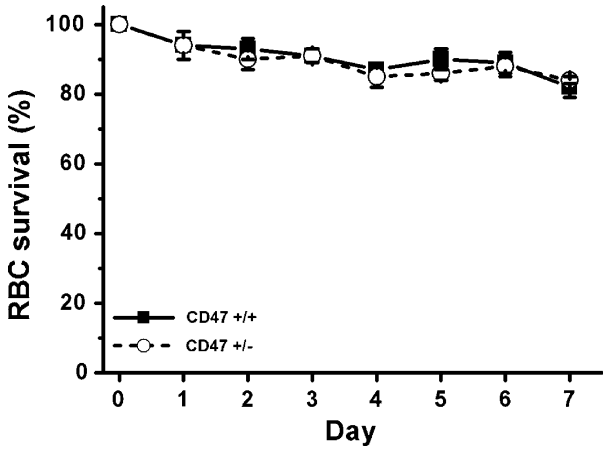

Fig. 2. Clearance of CD47$^{+/+}$ and CD47$^{+/-}$ RBCs in the absence of anti-RBC antibodies. CD47$^{+/+}$ recipient mice were injected with a 1:1 mixture of a total of 1×10^9 PKH67-labelled CD47$^{+/+}$ and PKH26-labelled CD47$^{+/-}$ RBCs. Clearance was followed in blood samples taken every 24 h, using flow cytometry. Values are means ± SEM from three mice.

In vitro phagocytosis of IgG-opsonized RBCs

We and others have previously shown that lack of CD47/SIRPα-interaction results in enhanced uptake of IgG-opsonized CD47$^{-/-}$ RBCs [2,14]. Thus, to further confirm a dose-dependent role of CD47 in regulating macrophage uptake of IgG-opsonized RBCs, RBCs from CD47$^{+/+}$, CD47$^{+/-}$ or CD47$^{-/-}$ mice were opsonized with a rabbit anti-mouse RBC antibody, which triggers Fcγ receptor-mediated phagocytosis in murine bone marrow-derived macrophages [2]. In agreement with that previously reported [2], phagocytosis of CD47$^{-/-}$ RBCs was significantly higher than that for equally opsonized CD47$^{+/+}$ RBCs (Fig. 4). Interestingly, phagocytosis of CD47$^{+/-}$ RBCs was also significantly higher than that for CD47$^{+/+}$ RBCs, but still significantly lower than that for CD47$^{-/-}$ RBCs (Fig. 4).

Discussion

In the present study, we show that about 50% reduction in the RBC CD47 expression level has a significant effect on antibody-mediated RBC clearance *in vivo*, and on macrophage phagocytosis of IgG-opsonized RBCs *in vitro*.

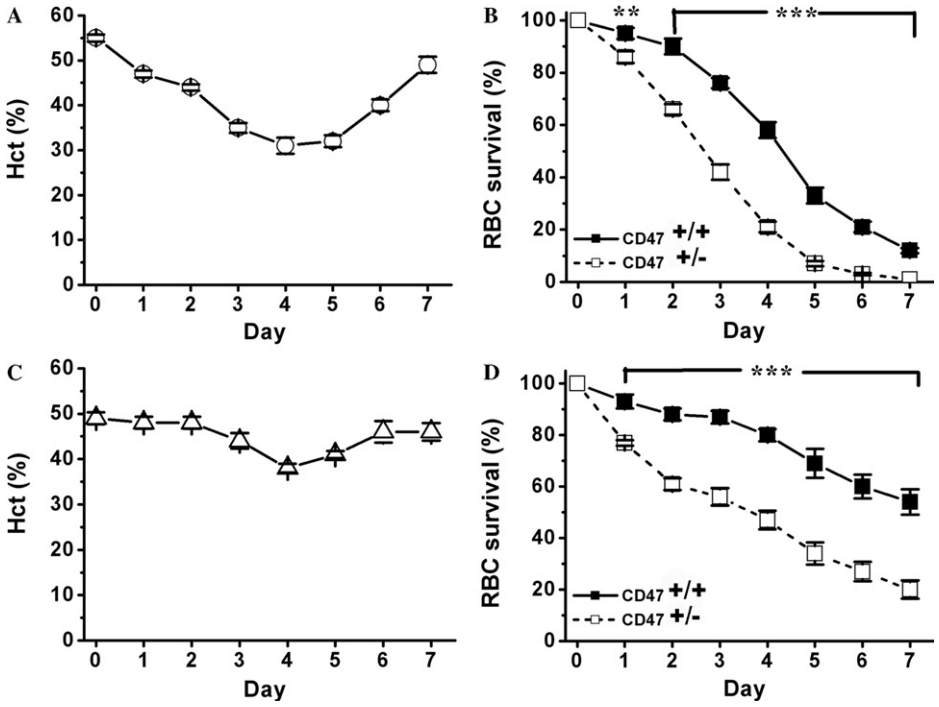

Fig. 3. Clearance of $CD47^{+/+}$ and $CD47^{+/-}$ RBCs in experimental AIHA. $CD47^{+/+}$ recipient mice were injected i.v. with PKH stained $CD47^{+/+}$ and $CD47^{+/-}$ RBCs, as described in the legend to Fig. 2, at 24 h before induction of AIHA. To induce AIHA, the pathogenic antibody 34-3C was injected i.p. at 3 μg/g (A,B) or 1 μg/g body weight (C,D) at day 0. Hct and clearance rate of injected RBCs were followed every 24 h, using flow cytometry. Values are means ± SEM from 7 mice (A,B) or 4 mice (C,D). $^{**}P < 0.01$ and $^{***}P < 0.001$, when comparing $CD47^{+/+}$ and $CD47^{+/-}$ RBCs at each time point, using Student's *t*-test for paired comparisons.

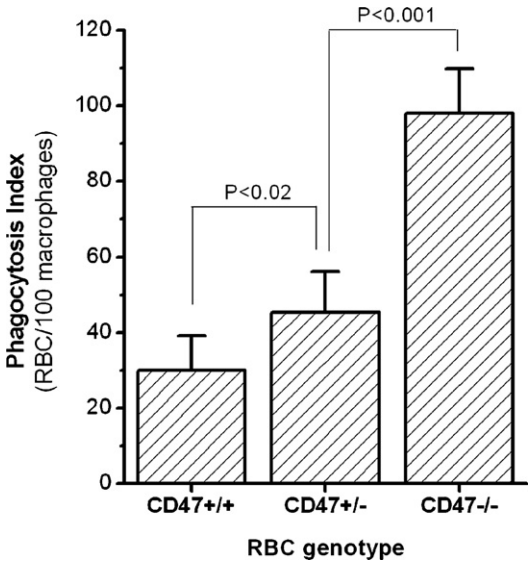

Fig. 4. *In vitro* phagocytosis of IgG-opsonized $CD47^{+/+}$, $CD47^{+/-}$, or $CD47^{-/-}$ RBCs. RBCs from $CD47^{+/+}$, $CD47^{+/-}$, or $CD47^{-/-}$ mice were equally opsonized with rabbit anti-mouse RBC IgG, and presented to bone marrow-derived macrophages for 20 min at 4 °C to allow for binding, and another 30 min at 37 °C to allow for phagocytosis. Non-ingested RBCs were lysed, and macrophages were stained with May-Grünewald/Giemsa. Phagocytosis index was calculated by counting the number of RBCs ingested/100 macrophages. Data are means ± SEM for 3 separate experiments. Statistical analysis was performed using Student's *t*-test for paired comparisons.

Knowledge on the mechanisms whereby RBC cell surface structures normally can protect against RBC phagocytosis by macrophages, could be important to improve RBC half-life in circulation following transfusion or in anemias. One such inhibitory mechanism is the finding of a low molecular weight phagocytosis-inhibitory factor (PIF), which was isolated from RBC membranes, and could inhibit non-specific, Fcγ receptor-mediated, or complement receptor-mediated phagocytosis [19]. Macrophage phagocytosis of blood cells is also controlled by interaction between CD47 on the blood cells and the inhibitory receptor SIRPα on the macrophage [2,8,15]. Complete lack of CD47 makes viable RBCs and platelets severely sensitive to elimination by macrophages *in vivo*, even in the absence of autoantibodies [2,7,8]. During storage of RBCs, loss of CD47 has been reported, although with different results. One study reported a loss of less than 10% of RBC CD47 after 28–48 days of storage [9], while another study detected more than 50% loss of CD47 after storage of RBCs for 24 days [10]. Although the discrepancy between these two studies may have several explanations, such as differences in storage medium or methods used to quantify RBC CD47, these findings clearly show that CD47 is lost from RBCs during storage. With this in mind, it becomes more important to understand at what level of CD47 reduction RBCs are more vulnerable to be cleared from the circulation to a greater extent. In the present study, we took advantage

of the fact that RBCs from $CD47^{+/-}$ mice express about 50% of the CD47 levels seen on RBCs from $CD47^{+/+}$ mice, to investigate the physiological importance of reduced CD47 expression on RBC survival *in vivo*. Our finding of an accelerated antibody-mediated clearance of $CD47^{+/-}$ RBCs already at a very low dose of anti-RBC antibodies, which resulted in a minor reduction in Hct, points at a significant effect when CD47 expression is reduced to about 50% of normal CD47 expression levels. These *in vivo* data were supported by *in vitro* phagocytosis experiments, showing that $CD47^{+/-}$ RBCs were taken up significantly more than equally opsonized $CD47^{+/+}$ RBCs, but still significantly less than $CD47^{-/-}$ RBCs.

During RBC ageing *in vivo* or during storage *in vitro*, changes to the RBCs take place, which are involved in regulating their final elimination by macrophages [16–18]. Although the exact mechanisms involved are still not completely understood, it is likely that a combination of changes in the RBC plasma membrane and its components, biomechanical changes in the RBC, and the presence of natural antibodies together create enough pro-phagocytic stimuli [16]. These pro-phagocytic stimuli may overcome the mechanisms that prevent phagocytosis (e.g. CD47/SIRPα interaction) during RBC interaction with macrophages. Although we did not detect an accelerated clearance of $CD47^{+/-}$ RBCs in the absence of RBC autoantibodies during the 7 day period used in our experiments, loss of CD47 during storage could shorten the half-life of RBCs in circulation, when any given pro-phagocytic stimulus is added to the system. Such pro-phagocytic stimuli may involve cell surface changes related to RBC ageing, i.e. binding of naturally occurring anti-RBC antibodies, which in turn can enhance macrophage RBC phagocytosis.

In conclusion, we have shown that a reduction of RBC CD47 expression levels to about 50% of normal can significantly shorten the half-life of RBCs in circulation in the presence of anti-RBC antibodies, and also increase Fcγ receptor-mediated phagocytosis of these RBCs *in vitro*. These findings may have importance for understanding the physiological significance of loss of RBC CD47 during storage, and in predicting the half-life of stored RBCs in circulation.

Acknowledgments

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