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Chapter 6

CD47 functions as a molecular switch for erythrocyte phagocytosis

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Abstract

CD47 on erythrocytes inhibits phagocytosis through interaction with the inhibitory immunoreceptor SIRPα expressed by macrophages. Thus, the CD47-SIRPα interaction constitutes a negative signal for erythrocyte phagocytosis. However, we report here that CD47 does not only function as a “don’t eat me” signal for uptake, but can also act as an “eat me” signal. In particular, a subset of old erythrocytes present in whole blood was shown to bind and to be phagocytosed via CD47-SIRPα interactions. Furthermore, we provide evidence that experimental ageing of erythrocytes induces a conformational change in CD47 that switches the molecule from an inhibitory signal into an activating one. Pre-incubation of experimentally aged erythrocytes with human serum prior to the binding assay was required for this activation. We also demonstrate that aged erythrocytes have the capacity to bind the CD47-binding partner thrombospondin-1 (TSP-1) and that treatment of aged erythrocytes with a TSP-1-derived peptide enabled their phagocytosis by human red pulp macrophages. Finally, CD47 on erythrocytes that had been stored for prolonged time was shown to undergo a conformational change and bind TSP-1. These findings reveal a more complex role for CD47-SIRPα interactions in erythrocyte phagocytosis, with CD47 acting as a molecular switch for controlling erythrocyte phagocytosis.
Introduction

Erythrocyte clearance has been studied for many years in the context of normal turnover\textsuperscript{1,2}, enhanced clearance due to defects in erythrocyte metabolism or changes in membrane composition\textsuperscript{3-5}, and in blood transfusion\textsuperscript{6}. A large number of factors that may influence erythrocyte clearance has been proposed, ranging from autoantibody binding to band 3 after conformational changes due to ageing (the “senescent cell antigen”)\textsuperscript{7,8}, to the expression of phosphatidylserine (PS) on the outer leaflet of the erythrocyte membrane\textsuperscript{9}. In general, it is believed that erythrocytes are cleared after accumulating so called “eat me” signals and are then phagocytosed by macrophages of the reticuloendothelial system (RES), predominantly in the spleen and liver\textsuperscript{1,10,11}. In contrast, one of the membrane proteins expressed by erythrocytes, CD47, has been shown to inhibit phagocytosis of erythrocytes by macrophages of the RES\textsuperscript{12-16}. CD47 exerts its inhibitory effect through binding to SIRP\textsubscript{α} on the macrophage, which induces inhibitory signaling by the immunoreceptor tyrosine-based inhibition motifs (ITIMs) residing in the cytoplasmic tail of SIRP\textsubscript{α}\textsuperscript{17,18}. Upon ligation of SIRP\textsubscript{α} by CD47 the SIRP\textsubscript{α} ITIMs recruit and activate tyrosine phosphatases SHP-1 and SHP-2, and this regulates, generally in a negative fashion, downstream signaling pathways and effector functions. The inhibitory effect of CD47 on erythrocyte clearance can be illustrated by transfusion of CD47-deficient erythrocytes into a wild-type recipient, which leads to rapid phagocytosis of the CD47-deficient erythrocytes by red pulp macrophages in the spleen\textsuperscript{12,19}. Thus, phagocytosis of erythrocytes is supposed to be the result of “eat me” signals already present that override the inhibitory signal of CD47. Although it has been suggested that the expression of CD47 decreases during in vitro erythrocyte ageing under blood bank conditions, there are other studies that suggest that CD47 levels remain unaltered\textsuperscript{20,21}. Nevertheless, it is well known that transfusion of red cell concentrates in patients leads to a rapid clearance of 10-25 \% of the transfused cells depending on storage time\textsuperscript{22,23}. This could contribute to some of the complications associated with blood transfusion, including transfusion-related acute lung injury (TRALI)\textsuperscript{24}. The mechanism behind this rapid clearance of a proportion of the red cells has not been clarified.

Intriguingly, evidence exists that the CD47-SIRP\textsubscript{α} interaction can also promote phagocytosis of apoptotic cells, although the mechanism behind this phenomenon is unknown\textsuperscript{25-26}. Furthermore, work by Kusakari et al. has indicated that SIRP\textsubscript{α} can mediate trans-endocytosis of CD47-containing membrane of adjacent cells\textsuperscript{27}, illustrating that SIRP\textsubscript{α} signaling can lead to other phenomena besides inhibition of phagocytosis. Based on these findings, we hypothesized that SIRP\textsubscript{α} might play a role in the removal of aged erythrocytes through CD47 binding. Using SIRP\textsubscript{α}-transfected cell lines we observed that aged erythrocytes bind to SIRP\textsubscript{α} through CD47. We obtained data that strongly suggest that CD47 undergoes a conformational change during ageing evoked by oxidative stress. Due to this conformational change, CD47 is then recognized, after binding of thrombospondin-1\textsuperscript{18,20}, as an “eat me” signal by SIRP\textsubscript{α}. We observed phagocytosis of experimentally aged erythrocytes by primary human red pulp macrophages through this mechanism, suggesting that this mechanism is operational in vivo. In line with our findings we observed that erythrocytes that had been stored for a prolonged period displayed a conformational change in CD47 when diluted into fresh whole blood, and subsequently bound thrombospondin-1. This result suggests a potential role for the CD47-SIRP\textsubscript{α} axis in the rapid phagocytosis of donor erythrocytes after transfusion. Collectively, our data identify CD47 as a
molecular switch for erythrocyte phagocytosis.

## Materials and methods

### Reagents

Mouse anti-human CD47 (clone B6H12)-allophycocyanin (APC), carboxyfluorescein diacetate succinimidyl ester (CFSE) and Iscove's modified Dulbecco medium (IMDM) were from Invitrogen (Pailey, United Kingdom). Mouse anti-human CD47 (clone 2D3)-fluorescein isothiocyanate (FITC) was obtained from eBioscience (San Diego, CA, USA). Streptavidin-APC was from BD Bioscience (Franklin Lakes, NJ, USA). Deoxyribonuclease II (DNase) and ascorbic acid were from Sigma-Aldrich (Saint Louis, MO, USA). Purified collagenase was obtained from Worthington (Lakewood, NJ, USA). Human serum albumin (HSA) and pasteurised plasma-protein solution (GPO) were obtained from Sanquin (Amsterdam, The Netherlands). Fetal calf serum (FCS), penicillin/streptomycin and L-glutamine were obtained from PAA (Pasching, Austria). DMEM/F12 culture medium was from Lonza (Cologne, Germany).

### Purification of erythrocytes from whole blood

Venous blood was collected from healthy donors, after obtaining informed consent. Blood studies were approved by the Sanquin Research institutional medical ethical committee in accordance with the standards laid down in the 1964 Declaration of Helsinki. Erythrocytes were isolated from fresh heparinized whole blood by centrifugation at 270g for 15 minutes. After removing the platelet-rich plasma and the peripheral blood mononuclear cells, the erythrocytes were washed 2 times with salineadenine-glucose-mannitol (150 mM NaCl, 1.25 mM adenine, 50 mM glucose, 29 mM mannitol) (SAGM) (Fresenius Kabi, The Netherlands) and resuspended in SAGM. Final cell concentration was determined with an Advia 2120 (Siemens Medical Solutions Diagnostics, Breda, The Netherlands).

### SIRPα constructs

Constructs were placed in pCDNA3 with BamHI and EcoRI restriction enzymes (Fermentas). A construct for full-length human SIRPα1 was transfected with Fugene (Roche) according to manufacturer’s protocol. A rat-human SIRPα fusion construct was generated as described elsewhere (Van Beek et al, submitted). In short, nt 1-1236 of rat SIRPα cDNA, counted from the first methionine, were fused to nt 1230-1509 of the human SIRPα cDNA. Amino acids 1-412 represent the extracellular domain of rat SIRPα and amino acids 411-503 represents the trans- and cytoplasmic domain of human SIRPα. The sequence of the construct was confirmed by sequencing. Rat SIRPα1 and mutated rat SIRPα1, namely SIRPα1-Δ87, SIRPα1 with all 4 tyrosines mutated and SIRPα1-V56M mutant, were also cloned by means of BamHI and EcoRI restriction enzymes and transfected with Fugene according to manufacturer’s protocol.

### Culturing and transfection of CHO cells

Chinese Hamster Ovary (CHO) cells were cultured in DMEM/F12 culture medium,
supplemented with 10 % (v/v) FCS, 1 % penicillin/streptomycin and 2 mM L-glutamine. The constructs were cloned by means of BamHI and EcoRI and put in pcDNA3 and transfected in CHO cells with fugene (Roche, Basel, Switzerland) according to the manufacturer’s protocol. Subsequently, the CHO cells were sorted on a BD FACS Aria II, (Becton Dickinson, Franklin Lakes, NJ, USA) as described below. CHO cells with human constructs were stained with an antibody recognizing SIRPα1 (mouse anti-human SIRPα, clone OX118). The rat constructs were detected with a mouse anti-rat SIRPα, clone ED9α. The CHO cells were incubated with 10 ng/ml primary antibody in HEPES buffer (132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄ [all from Sigma-Aldrich]) supplemented with 0.5 % (v/v) HSA, 1 mM CaCl₂ (Sigma-Aldrich) and 10 mM glucose (Sigma-Aldrich) (HEPES⁺) for 30 minutes on ice. After washing, the cells were incubated with 10 µg/ml goat anti-mouse IgG-alexa488 (Invitrogen) for 30 minutes on ice. The cells were washed and the cells with the highest mean fluorescence intensity were sorted and taken into culture. To keep the constructs in the CHO cells, 3.75 mg/ml Geneticin (Invitrogen) was added to the culture medium of transfected CHO cells.

**Binding of erythrocytes to CHO cells**

CHO cells were cultured in a 6-well plate (Corning Incorporated, Corning, NY, USA) to confluence, after which they were cultured for 2 more days. Erythrocytes isolated from fresh heparinized whole blood were washed and resuspended in HEPES⁺ to a final concentration of 2*10⁷ cells/ml. To block CD47 on the erythrocytes, the cells were resuspended to a final concentration of 1*10⁸ cells/ml in HEPES⁺ with 10 µg/ml CD47-F(ab’)² fragments (made from clone B6H12), after which they were washed and resuspended in HEPES⁺ to a final concentration of 2*10⁷ cells/ml. The CHO cells were washed with PBS, after which 4*10⁷ erythrocytes were added to 10 cm² dishes. The cells were incubated overnight in a 37 °C incubator. After washing the wells with PBS, erythrocyte binding was analyzed by wide field microscopy (Evos, AMG micro, Bothell, WA, USA).

**Confocal microscopy**

CHO cells were cultured as described above, with the following modifications. CHO cells were cultured in a 24-well plate with glass bottom (Zell Kontakt, NörtenHardenberg, Germany) instead of a 6-well plate. After the overnight incubation and removal of the unbound cells by washing, the cells were stained with 10 µg/ml unconjugated mouse anti-human glycophorin A (clone CD235a, Sanquin Reagents) in HEPES⁺. The cells were washed with HEPES⁺ and incubated with 10 µg/ml goat antismouse IgG-alexa488 in HEPES⁺. For intracellular staining of phagocytosed erythrocytes, all extracellular erythrocytes were lysed with lysis buffer, an ice-cold isotonic NH₄Cl solution (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 7.4). After 5 minutes incubation on ice and removing the supernatant, this step was repeated once. Thereafter the cells were fixed overnight in 4 % (w/v) ice-cold paraformaldehyde in phosphate-buffered saline (PBS). Subsequently, the cells were washed in PBS and incubated with 7 % bovine serum albumin (BSA, Sigma) in PBS for 30 minutes. The cells were washed in PBS and permeabilized with methanol/acetone (1:1 ratio, v/v) for 30 minutes. Staining was performed by incubating the permeabilized cells with 10 µg/ml unconjugated mouse IgG anti-human
glycophorin A in 7 % BSA. After washing, the cells were stained with goat anti-mouse IgG-alexa647 (Invitrogen). The cells were analyzed using a confocal microscope (Axiovert 100M, Zeiss, New York, NY, USA) and green events larger than 3 µm were counted by Image Pro-Plus version 6 (Media Cybernetics, Bethesda, MD, USA).

**CD47-bead coupling**

TransFluoSpheres carboxylate-modified microspheres of 1 µm (Invitrogen) were coated with the extracellular domain of CD47. This was done by diluting the beads in deionized water containing 2 mM NaN₃ to a final concentration of 3.3*10¹⁰ beads/ml. Subsequently, 24 mM streptavidin (Sigma-Aldrich) was added and this was incubated for 15 minutes at room temperature. After addition of 2.1 mM EDAC solution (7 mM N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloridein (EDAC)) in deionized water supplemented with 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES), pH 6) (all from Sigma-Aldrich) and adjusting the pH to 6.5, this was incubated for 2 hours at RT in the dark on a shaking block. To stop the reaction, 100 mM glycine was added and incubated for 30 minutes at room temperature. The beads were washed 3 times with PBS and resuspended in PBA (PBS supplemented with 0.5 % BSA and 0.02 % NaN₃ (w/v)) to a final concentration of 26.5*10⁶ beads/ml. Subsequently, 26 µg/ml biotinylated goat anti-human IgG antibody targeted against the Fcγ fragment (Jackson ImmunoResearch, West Grove, PA, USA) was added and incubated for 2 hours at 37 °C in a shaking heating block. The beads were washed twice with PBA and incubated overnight at 4 °C end-over-end after addition of 3.3 µg/ml CD47-fc chimera. The beads were washed 3 times with PBA, resuspended to 1*10⁹ beads/ml in PBA and stored at 4 °C until use.

**Bead binding assay**

CHO cells were cultured as described above for confocal microscopy. The wells were washed with PBS and 1 ml HEPES⁺ was added to each well. Subsequently, 5*10⁶ beads were added to each well and incubated for 30 minutes in a 37 °C incubator. The wells were washed thoroughly with PBS to remove unbound beads. Subsequently, bead binding was analysed by fluorescence microscopy (Evos) and bound beads were counted.

**CD47 analysis after experimental ageing**

Conformational changes of CD47 were analyzed with an APC-conjugated conformation independent antibody (clone B6H12) and a FITC-conjugated conformation-dependent antibody (clone 2D3). Experimental ageing was induced by incubating erythrocytes with CuSO₄ and ascorbic acid as described below. After washing the cells, a double-staining was performed with 10 µg/ml of both CD47 antibodies diluted in HEPES⁺. The samples were washed and analyzed with a flow cytometer with high throughput system (LSRII, Becton Dickinson) and analyzed with computer software (FACSDiva version 6.1, Becton Dickinson).

**TSP-1 peptide binding and staining for flow cytometry**

Binding of the TSP-1 peptide was analyzed as follows. Erythrocytes, untreated or after
induction of oxidative damage, were resuspended in HEPES\(^+\) and 50 µg/ml biotinylated TSP-1 peptide (4N1K, amino acid sequence; KRFYVVMWKK) or 50 µg/ml biotinylated control peptide (4NGG, amino acid sequence; KRFYGGMWKK) was added. Subsequently, the cells were incubated for 30 minutes on ice, washed and incubated for 30 minutes with 1:250 steptavidin-APC on ice. After washing the cells and resuspending in HEPES\(^+\) the cells were analyzed by a flow cytometer with high throughput system and analysed with computer software.

**Thrombospondin-1 binding**

Thrombospondin-1 binding was analysed by staining with the mouse anti-human thrombospondin antibody A6.1 (Abcam, Cambridge, MA, USA). This was done by washing and diluting erythrocytes with HEPES\(^+\) to a final concentration of 1*10\(^8\) cells/ml and incubating them with 10 µg/ml A6.1. Subsequently, the cells were incubated for 30 minutes on ice, washed and incubated with 10 µg/ml goat anti-mouse IgG-alexa488 for 30 minutes on ice. After washing the cells and resuspending in HEPES\(^+\) the cells were analyzed by a flow cytometer with high throughput system and analysed with computer software.

**Experimental ageing of erythrocytes**

Erythrocytes isolated from whole blood were spun down and resuspended in PBS supplemented with 0.2 mM CuSO\(_4\) and 5 mM ascorbic acid to a final concentration of 0.4*10\(^8\) cells/ml\(^15\). After incubating the cells at 37 °C for 60 minutes in a shaking heating block the cells were washed 3 times with PBS and resuspended in HEPES\(^+\).

**Experimental ageing of CD47-beads**

To induce oxidative damage in CD47-coupled beads, CD47 beads were diluted to a final concentration of 0.3*10\(^8\) beads/ml in PBS supplemented with 0.2 mM CuSO\(_4\) and 5 mM ascorbic acid. After incubating the beads at 37 °C for 60 minutes in a shaking heating block the beads were washed 3 times with PBS and resuspended in HEPES\(^+\).

**Isolating spleen macrophages**

Human spleen macrophages were isolated from samples of human spleen. The sample of about 10 cm\(^3\) was placed in a glass container with HEPES buffer supplemented with 0.5 µg/ml tirofiban (Aggrastat; Merck, Whitehouse Station, NJ, USA), 100 U/ml collagenase, 1 mM CaCl\(_2\) and 200 U/ml DNase. The spleen sample was cut into small pieces and a stirring magnet was placed in the container. After incubating for 2.5 hours at 37 °C while stirring, the cell suspension was put on a 100-µm filter. The filter was flushed with 30 ml PBS supplemented with 0.38 % sodium citrate and 10 % pasteurized plasma-protein solution (PBS/TNC/GPO). The suspension was carefully pipetted onto a Percoll layer (GE Healthcare, Waukesha, WI, USA) with a density of 1.076. After centrifugation for 20 minutes at 960g, the ring fraction was collected and washed twice with PBS/TNC/GPO, after which the cells were resuspended in phenol-red free IMDM medium (Invitrogen), supplemented with 10 % fetal calf serum, 1 % penicillin/streptomycin (v/v) and 2 mM L-glutamine (culture medium). Subsequently, the cells were counted on a Coulter Casy counter (Beckman Coulter, Indianapolis, IN, USA) and cultured at 4*10\(^6\) cells/ml
in a 75 cm² culture flask with filter cap (TPP, Trasadingen, Switzerland). After culturing for 2
days, the cells were washed with PBS and fresh culture medium was added.

**Phagocytosis assay**

One day before the phagocytosis assay, THP-1 cells, cultured in phenol-red free IMDM
medium (Invitrogen), supplemented with 10 % (v/v) fetal calf serum, penicillin/streptomycin
and 2 mM L-glutamine, were washed once with PBS and resuspended in culture medium to a
final concentration of 1*10⁶ cells/ml. The THP-1 cells are a human acute monocytic leukemia
cell line. Subsequently, 50 ng/ml PMA was added and the cells were incubated for 4 hours
in a 37 °C incubator. After this incubation, the cells were washed with PBS, resuspended in
culture medium and 250,000 cells were plated per well in a 12-well flat bottom plate (Corning
Incorporated, Corning, NY, USA) and cultured overnight in an incubator.

Spleen macrophages were detached by trypsinization. After the cells had detached, they
were collected, washed with PBS and resuspended in culture medium. Subsequently, 250,000
cells were plated per well in a 12-well flat bottom plate and cultured overnight in an incubator.

Erythrocytes isolated from whole blood or after induction of experimental ageing were
washed with HEPES⁺ and diluted to a final concentration of 2*10⁸ cells/ml in HEPES⁺. Subsequently, 25 µM CFSE was added and the cells were incubated at 37 °C for 30 minutes end-
over-end. After the cells had been spun down and resuspended in PBS to a final concentration
of 2*10⁸ cells/ml, they were incubated at 37 °C for 30 minutes end-over-end.

After washing the cells with HEPES⁺, the cells were resuspended to a concentration of
10⁸ cells/ml. The THP-1 cells and spleen macrophages were washed with PBS and resuspended
in 1 ml of HEPES⁺. To each well of macrophages, 2.5*10⁶ erythrocytes were added and incubated
for 2 hours in a 37 °C incubator.

After incubation, the supernatant was removed from each well and lysis buffer was added to
lyse non-phagocytosed erythrocytes. This step was repeated once after 5 minutes incubation on
ice and removing the supernatant. Subsequently, PBS supplemented with 10 mM EDTA (Merck)
and 80 mM lidocaine mono-hydrate (Sigma-Aldrich) was added to detach the cells. The cells
were collected, washed with and resuspended in HEPES⁺, and analyzed on a flow cytometer
(Becton Dickinson) with high throughput system. Data analysis was performed with FACSDiva
software (Becton Dickinson).

**Whole blood dilution**

Erythrocytes obtained from a stored red cell concentrate (RCC), prepared as described
elsewhere³¹, were washed twice with HEPES buffer and diluted to a hematocrit of 40 %. The
erthrocytes were diluted 1:10 with heparinized whole blood obtained from AB⁺ donors, and
incubated overnight at 37 °C. To detect the donor erythrocytes, the cells were stained with
antibodies against the minor blood group antigen s (Ortho Clinical Diagnostics, Tilburg, The
Netherlands). This was done by diluting the blood to a final concentration of 1*10⁶ cells/ml
in HEPES⁺ and adding undiluted human anti-human s in a ratio of 5:1. After incubation for
30 minutes at room temperature, the cells were washed extensively before re-suspending in
10 µg/ml goat anti-human IgG-alexa568 (Invitrogen) to a final concentration of 1*10⁷ cells/
ml and incubating for 30 minutes at room temperature. The cells were subsequently washed
and resuspended in HEPES* and analyzed on a flow cytometer (Becton Dickinson) with high throughput system. Data analysis was performed with FACSDiva software (Becton Dickinson).

**Statistical analysis**

Data was analyzed using Graphpad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA). Statistical analysis was performed by t-test when 2 groups were compared or one-way ANOVA with Tukey’s Multiple Comparison Test when more than 2 groups were compared.

**Results**

**SIRPα transfected CHO cells bind erythrocytes from whole blood**

To identify a possible interaction between CD47, expressed on erythrocytes, and SIRPα, we transfected CHO cells with full-length human SIRPα. Next, we incubated erythrocytes overnight with the CHO cells. We observed that, after thorough washing, a small fraction of the erythrocytes was bound to the CHO SIRPα cells (figure 1a). We did not observe binding of erythrocytes to untransfected CHO cells (figure 1a). To establish that the observed interaction between the erythrocytes and the CHO SIRPα cells was mediated through CD47 on the erythrocytes and SIRPα on the CHO cells, we preincubated the erythrocytes with a CD47 blocking antibody, B6H12 (figure 1a). The CD47 blocking antibody inhibited the binding of the erythrocytes to the CHO SIRPα cells. In addition, pre-treatment of the CHO SIRPα cells with a blocking antibody against SIRPα (SE7C2) also completely inhibited binding of erythrocytes to the CHO SIRPα cells (figure 1a), thus implicating that the binding of the erythrocytes to the CHO SIRPα cells was mediated by CD47-SIRPα interactions. To gain more insight into the nature of the erythrocytes with SIRPα binding capacity, we separated the erythrocytes on the basis of their density into younger and older cells by Percoll fractionation32 and compared
their binding to CHO SIRPα cells. Strikingly, the fraction containing the youngest erythrocytes did not show any binding to the CHO SIRPα cells, while the fraction containing the oldest cells showed the highest amount of erythrocytes bound (figure 1b), indicating that only a small fraction of the aged erythrocytes have the capacity to bind SIRPα through CD47.

**CHO SIRPα cells bind and phagocytose erythrocytes**

To determine whether bound erythrocytes were phagocytosed by CHO SIRPα cells we performed live imaging of CHO SIRPα cells that had bound erythrocytes. This demonstrated that a significant proportion of the erythrocytes that had been bound by the CHO SIRPα cells was phagocytosed (Supplementary video 1). Furthermore, when after an overnight incubation, bound erythrocytes were lysed and the CHO SIRPα cells were permeabilized and intracellularly
stained for glycophorin A, phagocytosed erythrocytes were detected by confocal microscopy (figure 2a).

SIRPα binding of CD47 on aged erythrocytes is dependent on the SIRPα cytoplasmic tail

We investigated whether the binding of aged erythrocytes to SIRPα was dependent on the intracellular tail of SIRPα. We expressed several mutants of SIRPα in CHO cells, including a mutant in which the four tyrosine residues of the ITIM motifs (tyrosine mutant) were mutated to phenylalanine. Another mutant lacked 87 amino acids of the C-terminus of SIRPα (Δ87 mutant), and therefore has only a small cytoplasmic domain of approximately 23 amino acids remaining. Also, the V56M point mutation within the N-terminal V-set Ig domain that abolishes CD47 binding, as shown by us and others33,34, was tested for its ability to bind old erythrocytes through CD47 recognition. These mutants were expressed at a similar level as the SIRPα construct they were derived from (figure 2b). The mutants displayed non-significant differences in binding of recombinant human CD47 coupled to beads compared to wild-type SIRPα (figure 2c), except the V56M mutant, which did not display binding of CD47 beads above background level. Furthermore, the binding of erythrocytes freshly isolated from whole blood to the tyrosine mutant was not significantly different from the binding to the construct with the wild-type intracellular tail (p<0.05) (figure 2d). However, both the Δ87 mutant as well as the V56M mutant, showed significantly reduced binding of erythrocytes in comparison with the construct with the wild-type intracellular tail (p<0.05) (figure 2d). So, in contrast to binding of beads coated with CD47, deletion of part of the cytoplasmic tail of SIRPα affects the binding of aged erythrocytes to SIRPα.

CD47 on experimentally aged erythrocytes is recognized by SIRPα.

During the erythrocyte lifespan, oxidative damage accumulates on erythrocyte membrane proteins, which contributes to their ageing and their subsequent clearance. We therefore investigated whether erythrocytes that were aged artificially by oxidation would acquire the capacity to bind SIRPα. For this purpose young erythrocytes (obtained by density centrifugation), which possess minimal SIRPα-binding capacity, were used. When the erythrocytes were incubated with the CHO SIRPα cells after oxidation, no binding was observed (figure 3a). However, when the experimentally aged erythrocytes were preincubated with human serum, substantial binding did occur (figure 3a). Based on these finding we hypothesized that oxidation induces a conformational change in CD47 that would lead to binding of a serum factor that would subsequently support the binding of SIRPα. First, we tested whether CD47 undergoes conformational changes due to oxidation. Two antibodies directed against different CD47 epitopes, used previously to detect potential conformational differences in CD47 on sickle erythrocytes, were employed to detect differences in CD47 conformation upon ageing. One of the antibodies, B6H12, recognizing a constant epitope, showed similar levels of binding to oxidized and non-oxidized erythrocytes (figure 3b), indicating that the total level of surface expression was not altered between these erythrocyte preparations. Strikingly, binding of the second antibody, 2D3, which has previously been shown to recognize a variable epitope on CD47, showed enhanced binding to oxidized erythrocytes, indicating that CD47, or at least
Figure 3: CD47 on experimentally aged erythrocytes is recognized by SIRPα.
(a) Experimental ageing of erythrocytes in combination with serum treatment induces binding to CHO SIRPα cells. (b) CD47 staining with the conformation independent antibody B6H12 and 2D3, an antibody that detects a conformation dependent epitope of CD47 on experimentally aged erythrocytes (green), shows a conformational change of CD47 after experimental ageing when compared to untreated erythrocytes (purple). (c) Experimental ageing of erythrocytes results in binding of the TSP-1-derived peptide 4N1K. No binding was observed for the control peptide, 4NGG. Furthermore, no streptavidin-APC staining was observed for fresh erythrocytes and oxidized erythrocytes when no peptide was added or when fresh erythrocytes were incubated with the 4NGG peptide (data not shown). (d) Ageing of young erythrocytes in combination with 4N1K peptide incubation results in binding to CHO SIRPα cells. (e) Oxidation of CD47 beads inhibits binding to CHO SIRPα cells. The binding of oxidized CD47 beads could subsequently be induced in combination with the 4N1K peptide, but not with the control peptide, 4NGG. Data shown are representative of 3 experiments.
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part of the CD47 molecules present on erythrocytes, had indeed undergone a conformational change (figure 3b).

One of the best known binding partners of CD47 is thrombospondin-1 (TSP-1), a protein derived from thrombocytes. Interestingly, TSP-1 binding to CD47 on erythrocytes induces erythrocyte death, a response that can be mimicked by a C-terminal peptide derived from TSP-1, 4N1K. Moreover, changes in CD47 conformation on sickle cell disease erythrocytes induce binding of TSP-1 to CD47. Control and oxidized erythrocytes were incubated with either biotinylated 4N1K peptide or a biotinylated control peptide, 4NGG. Experimentally aged erythrocytes bound the 4N1K peptide, but not the 4NGG peptide, indicating that the conformational change induced by experimental ageing results in increased binding of 4N1K to CD47 (figure 3c). Next, the ability of the 4N1K peptide to induce binding of experimentally aged erythrocytes to CHO SIRPα cells was tested. Oxidized erythrocytes were incubated with either the 4NGG peptide or the 4N1K peptide. Only when the 4N1K peptide was used, binding was observed (figure 3d). In contrast, untreated erythrocytes did not bind to CHO SIRPα cells, even when they were incubated with the 4N1K peptide (data not shown).

To investigate if the conformational change in CD47 induced by oxidative damage represents a direct effect on the extracellular domain of CD47 or an indirect effect via other membrane proteins present in the CD47 complex which might be oxidized, beads coated with the extracellular domain of CD47 were oxidized and binding to CHO SIRPα cells was tested. When the beads were not oxidized, 4N1K and 4NGG had no effect on bead binding to CHO SIRPα cells (figure 3e). However, after oxidation the beads were unable to bind to the CHO SIRPα cells. The binding to CHO SIRPα cells was restored by the pre-incubation with the 4N1K peptide, but not by the 4NGG peptide. We did not observe any binding to CHO cells which were not transfected with SIRPα (data not shown).

Experimentally aged erythrocytes are phagocytosed by human macrophages after 4N1K opsonization.

To investigate whether the interaction between CD47 on experimentally aged erythrocytes and SIRPα can also trigger phagocytosis by human macrophages we first tested the ability of the human monocytic cell line THP-1 to phagocytose experimentally aged erythrocytes. The inhibitory role of CD47 on erythrocyte phagocytosis by THP-1 can be demonstrated by pre-treating the erythrocytes with F(ab’)_2 fragment of the B6H12 antibody directed against CD47 that blocks CD47-SIRPα interactions. As anticipated, erythrocytes were phagocytosed when the interaction between CD47 and SIRPα was blocked by the F(ab’)_2 fragment directed against CD47 (figure 4a), indicating that CD47 normally functions as a “don’t eat me” signal on erythrocytes. However, when we incubated experimentally aged erythrocytes after opsonization with the 4NGG peptide or the 4N1K peptide with the THP-1 cells, only erythrocytes that were both oxidized and incubated with the 4N1K were phagocytosed, strongly suggesting that under these conditions, the CD47-SIRPα interaction is changed in such a manner that phagocytosis is promoted (figure 4a).

Next, we tested the cell type that is responsible for the uptake of aged erythrocytes in vivo, the splenic red pulp macrophage (RPM) (figure 4b). Human RPM, like their murine counterparts, express SIRPα (figure 4c). As for the THP-1 cells, we determined whether the CD47-SIRPα
interaction between normal erythrocytes and spleen macrophages mimics that found in the murine system. As expected, we observed very little phagocytosis of fresh erythrocytes when incubated with human red pulp macrophages (RPM) (figure 4d). Again, erythrocytes were effectively phagocytosed when they were pre-treated with the F(ab')₂ fragment directed against CD47. Furthermore, experimentally aged erythrocytes opsonized with the 4N1K peptide were phagocytosed, whereas 4NGG peptide treated cells were not, illustrating that primary human RPM are able to phagocytose erythrocytes in a CD47-SIRPα-mediated fashion.

**Prolonged storage of erythrocytes induces a conformational change in CD47 and leads to TSP-1 binding**

For transfusion it is accepted that up to 25 % of the transfused cells is removed from the circulation in the first 24 hrs after transfusion²². Since we found that aged erythrocytes bound to CHO SIRPα cells and macrophages in a CD47-SIRPα-dependent fashion, we hypothesized that
this mechanism might explain the loss of donor erythrocytes after transfusion. To determine changes in the conformation of CD47 and subsequent TSP-1 binding during red cell storage, we sampled stored erythrocytes during a period of 35 days. However, very little phenotypic changes were observed when different antigens on erythrocytes were analyzed in samples taken directly from the storage bag (P. Burger, unpublished). This was also true for the conformation of CD47. In particular, we did not observe any changes in the ratio between the binding of the B6H12 and the 2D3 antibody or any binding of TSP-1 protein (data not shown). Therefore, we mimicked the conditions of a blood transfusion ex vivo by diluting donor erythrocytes in freshly drawn whole blood of an AB⁺ recipient in a 1:10 ratio and incubated this overnight at 37 °C. The donor cells were then identified by detection of minor blood group antigens in the mixture and analyzed for binding of the two different CD47 antibodies as well as TSP-1 binding. Erythrocytes stored for 35 days displayed increased binding of the 2D3 antibody compared to the recipient erythrocytes (figure 5a). In line with our previous finding that experimentally aged erythrocytes, which also have an increase in the expression of the 2D3 epitope and bound the 4N1K peptide, the 35 days stored erythrocytes bound plasma-derived TSP-1 (figure 5b). In addition, the 4N1K peptide bound the donor cells as well, while the control peptide, 4NGG,
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did not (figure 5c). These data strongly suggest that upon transfusion at least part of the donor cells will bind TSP-1 due to conformational changes in CD47, which ultimately may lead to their clearance. To confirm that the 4N1K peptide indeed bound to CD47 present on the erythrocyte membrane, we performed a competition assay between the 4N1K peptide and both CD47 antibodies, B6H12 and 2D3. Therefore, 35 days stored erythrocytes were first incubated with the CD47 antibodies or the biotinylated TSP-1 peptides. Subsequently, after incubation with one of the CD47 antibodies, the erythrocytes were incubated with either the biotinylated 4N1K or biotinylated 4NGG peptides. Erythrocytes that were incubated with the biotinylated TSP-1 peptides were subsequently incubated with either B6H12 or 2D3. After these incubations total 4N1K or 4NGG binding was determined by streptavidin binding. When we first incubated with the 4N1K peptide and subsequently with the CD47 antibodies, we observed that B6H12 could compete with the 4N1K peptide for binding to CD47 (figure 5d). The 2D3 antibody did not compete with the 4N1K peptide. However, when we first incubated with the B6H12 or 2D3 antibodies and subsequently with the 4N1K peptide, we observed no binding of 4N1K indicating that the affinity of the CD47 antibodies for CD47 is higher than the 4N1K peptide.

Discussion

Although CD47-SIRPα interaction is well-known for its ability to inhibit phagocytosis of CD47 expressing cells12,19, there have been several reports that provide evidence for a role for CD47 and SIRPα in promoting uptake of apoptotic cells25,26. Here we describe the recognition and uptake of aged erythrocytes through CD47-SIRPα interaction as a new mechanism for erythrocyte clearance.

We obtained data that support a model in which a conformational change in CD47 is induced during ageing, after which TSP-1 binds to CD47 and hereby creates a new binding site for SIRPα, which is then able to recognize CD47 as an “eat me” signal instead of a “don’t eat me” signal. Importantly, oxidation of erythrocytes induced the conformational change in CD47 that was required for TSP-1 binding, suggesting that oxidative damage can be an important factor in generating this “eat me” signal on erythrocytes. The same oxidation abolished binding of CD47-coated beads to SIRPαCHO cells, suggesting that the conformational change observed in erythrocytes is primarily a direct effect on CD47 rather than an effect of oxidation of other membrane proteins. However, although the oxidation of beads abolished binding, we did not observe increased phagocytosis of erythrocytes after inducing experimental ageing. This suggests that at least part of the CD47 present on aged erythrocytes can still interact with SIRPα, or that only part of the CD47 is modified by oxidation. As for CD47 and SIRPα, TSP-1 has been described to promote phagocytosis of apoptotic cells40. We were able to confirm that oxidation of erythrocytes and subsequent incubation with the TSP-1 peptide leads to uptake by phagocytes, including THP-1 cells and primary human splenic RPM. The results with the THP-1 cells as well as the splenic RPM indicate that oxidation alone, and the subsequent conformational change in CD47, do not lead to phagocytosis of the erythrocytes (figure 4a and b). This means that in the absence of the TSP-1 peptide, CD47 still delivers its inhibitory signal; blocking CD47 interaction with SIRPα through F(ab’)2 fragments leads to phagocytosis of erythrocytes, indicating that this signal is crucial in inhibiting phagocytosis of erythrocytes. After incubation with the TSP-1
peptide phagocytosis is observed, indicating that erythrocytes are taken up only after CD47 has changed conformation and has bound TSP-1. Intriguingly, TSP-1 has also been shown to be able to induce erythrocyte PS exposure through CD47 after prolonged incubation38. We therefore propose a model in which the conformational change in CD47 and subsequent TSP-1 binding are at the basis of the recognition of aged erythrocytes as well as the generation of additional “eat me” signals.

How TSP-1 is able to induce phagocytosis of old erythrocytes through binding to CD47 and subsequent recognition by SIRPα is currently unknown. Although the results from the phagocytosis of aged erythrocytes treated with the TSP-1 peptide might suggest that TSP-1 is simply blocking the CD47-SIRPα interaction, this is not in line with the results from the CHO SIRPα binding. We clearly observed that TSP-1 was able to induce binding of aged erythrocytes to CHO SIRPα cells, which could not occur if TSP-1 would be blocking the CD47-SIRPα interaction. In line with this are the observations with oxidized beads coated with the extracellular domain of CD47. Upon oxidation, the CD47-coated bead binding to CHO SIRPα cells was abolished. However, when the oxidized beads are pre-treated with the 4N1K peptide we observed again binding to CHO SIRPα cells, but not to CHO cells which were not transfected with SIRPα. This suggests that TSP-1 does not block the interaction of CD47 with SIRPα, but rather alters the interaction. TSP-1 has to bind close to the epitope of B6H12 on the Ig domain of CD47, as shown by the competition between B6H12 and the TSP-1 peptide. We therefore propose that TSP-1 binds to CD47 and thereby either alters the conformation of CD47 even further allowing new binding sites to bind to SIRPα. It seems also possible that TSP-1 fixes CD47 in a conformation so that it is differently recognized by SIRPα. Further structure evidence will be necessary to give more insight into this interaction.

Our data indicate that CD47 regulates the recognition of erythrocytes that are destined to be cleared by macrophages in the spleen. We speculate that this mechanism may also be relevant for other cell types. In line with this thinking are previous publications in which CD47 on other cell types has also been indicated to be necessary for efficient phagocytosis of apoptotic cells25,26.

The signal transduction cascade that SIRPα uses to transmit inhibitory signals is well known. The cytoplasmic region of SIRPα contains four ITIMs, which upon ligand binding can become phosphorylated, and mediate recruitment and activation of the tyrosine phosphatases SHP-1 and SHP-227. SHP-1 and SHP-2 can, in turn, dephosphorylate specific protein substrates and thereby regulate phagocytosis in a negative fashion. However, it is at present completely unknown how SIRPα signaling would lead to positive regulation of phagocytosis. Transfection of SIRPα in CHO cells (figure 2a and supplementary video) was sufficient for phagocytosis of aged erythrocytes, indicating that the signaling molecules required for SIRPα dependent phagocytosis are present in these cells. CHO cells have been described to express SHP-1 and SHP-221,42 providing the possibility that these proteins also regulate phagocytosis through SIRPα. Currently, the signal transduction cascade leading to phagocytosis after SIRPα signaling is under investigation in CHO cells as well as in primary human RPM.

The data presented here may explain the enhanced clearance of erythrocytes in a number of circumstances. First, erythrocytes that have been stored for several weeks undergo a conformational change in CD47 when incubated in fresh blood and bind TSP-1, which may explain their high rate of clearance after transfusion (figure 5). Second, sickle erythrocytes also display an increase in the binding of the conformation-dependent CD47 antibody 2D3
and bind TSP-1\(^{43}\). This finding may explain in part the enhanced uptake of sickle erythrocytes in the spleen. Although it seems clear that changes in CD47 conformation may be a critical determinant for erythrocyte survival, it is currently not known which phenomena drive this CD47 conformational switch and whether this occurs during normal red cell ageing in vivo. As the conformational change in CD47 was only observed after incubation of long stored erythrocytes in whole blood, it is likely that other plasma components first induce the conformational change after which TSP-1 is able to bind to the changed CD47. As part of the band 3 complex in the erythrocyte membrane, it is also well possible that the change in CD47 that leads to the “eat me” configuration is due to modifications in band 3, i.e. phosphorylation\(^{44}\), cleavage and/or modification by transglutaminases\(^{45}\) that may occur during ageing. In line with this are the observed differences in binding of erythrocytes and beads coated with the extracellular domain of CD47 to CHO cells transfected with SIRP\(\alpha\) mutants. As the CD47 extracellular domain on the erythrocytes and beads is similar, this suggests that the transmembrane or intracellular part of CD47 also plays an important role in recognition by SIRP\(\alpha\). As CD47 is in a major protein complex with among others band 3, glycophorin A and CD44, an indirect effect on CD47 conformation by modification of other membrane proteins is well possible.

Another possibility is that CD47 becomes clustered on the erythrocyte membrane, due to increased mobility, can then bind TSP-1 and is subsequently recognized by SIRP\(\alpha\) as an “eat me” signal. Clustering of CD47 has been shown to occur on apoptotic erythrocytes\(^{46}\), but the contribution of this phenomenon to inhibition or facilitation of clearance through SIRP\(\alpha\) remains unknown. These different possibilities are currently under investigation.

### Supplementary video

http://bloodjournal.hematologylibrary.org/content/119/23/5512/suppl/DC1

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