Physiologically aged red blood cells undergo erythrophagocytosis *in vivo* but not *in vitro*

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ABSTRACT

Background
The lifespan of red blood cells is terminated when macrophages remove senescent red blood cells by erythrophagocytosis. This puts macrophages at the center of systemic iron recycling in addition to their functions in tissue remodeling and innate immunity. Thus far, erythrophagocytosis has been studied by evaluating phagocytosis of erythrocytes that were damaged to mimic senescence. These studies have demonstrated that acquisition of some specific individual senescence markers can trigger erythrophagocytosis by macrophages, but we hypothesized that the mechanism of erythrophagocytosis of such damaged erythrocytes might differ from erythrophagocytosis of physiologically aged erythrocytes.

Design and Methods
To test this hypothesis we generated an erythrocyte population highly enriched in senescent erythrocytes by a hypertransfusion procedure in mice. Various erythrocyte-aging signals were analyzed and erythrophagocytosis was evaluated *in vivo* and *in vitro*.

Results
The large cohort of senescent erythrocytes from hypertransfused mice carried numerous aging signals identical to those of senescent erythrocytes from control mice. Phagocytosis of fluorescently-labeled erythrocytes from hypertransfused mice injected into untreated mice was much higher than phagocytosis of labeled erythrocytes from control mice. However, neither erythrocytes from hypertransfused mice, nor those from control mice were phagocytosed *in vitro* by primary macrophage cultures, even though these cultures were able to phagocytose oxidatively damaged erythrocytes.

Conclusions
The large senescent erythrocyte population found in hypertransfused mice mimics physiologically aged erythrocytes. For effective erythrophagocytosis of these senescent erythrocytes, macrophages depend on some features of the intact phagocytosing tissue for support.

Key words: red blood cell, senescence, erythrophagocytosis.

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The online version of this article has a Supplementary Appendix.

Introduction

Red blood cells (RBC) have a life span of 120 days in humans and about 45 days in mice.[1] Removal of senescent RBC (sRBC) from the circulation occurs through phagocytosis (erythrophagocytosis, EPC), which takes place mainly in macrophages of the spleen, but also in the liver and the bone marrow.[2] Macrophages recognize sRBC by a range of senescence markers.[3] Following recognition and binding, the RBC that display senescence markers are phagocytosed, undergo hemolysis and their components are reutilized.

Aging of RBC includes changes in many properties: decreased metabolic activity, morphological alterations, including decreased cell volume and changes in cell shape, and quantitative and qualitative modulations of the surface.[3] Decreased metabolic activity is manifested by loss of aspartate amino transferase[4] and esterase[5] activities. Surface modulations include external exposure of membrane phosphatidylserine (PS), decreased membrane fluidity, decreased levels of sialic acid and CD47 and binding of autologous immunoglobulins and opsonins.[3,6-13] In addition, RBC are naturally damaged by oxidation during aging and a number of molecular modifications induced by oxidative stress such as PS externalization have been observed in sRBC.[3,14] Some of these modulations are recognized by macrophages as senescence signals but the relative contribution of these signals to EPC is not known.

In vitro experiments have been widely used to study the senescence markers of RBC, the mechanism of EPC and its effect on macrophages.[15-18] These studies mainly utilized primary cultures of macrophages derived from peripheral monocytes, the peritoneum or the bone marrow. The RBC were treated with chemicals or antibodies to induce the expression of individual aging parameters. These damaged RBC mimicked the naturally *in vivo* aged sRBC and were phagocytosed *in vitro*. Glutaraldehyde[10] or ribavirin[19] was used to damage the RBC morphology.[9] External exposure of PS was mostly induced by calcium and calcium-ionophore administration,[15,20] but was also detected after the addition of ribavirin, oxidizing agents, low levels of lead or prolonged incubation in phosphate-buffered saline (PBS).[21,22] The naturally occurring accumulation of anti-band 3 antibodies on RBC membranes during aging was mimicked successfully by opsonization of RBC, which led to massive EPC.[23-26] Chemical oxidation by addition of hydrogen peroxide, tert-butyl hydroperoxide or copper with ascorbate was also used to damage RBC and these damaged RBC were used to mimic sRBC in EPC assays.[27-30] In addition, RBC from mice with targeted deletion of CD47 were shown to be phagocytosed more readily *in vivo* and *in vitro* than normal RBC.[31]

Another approach to characterize sRBC used naturally aged RBC that were isolated by separation of RBC on a Percoll gradient, using the principle that RBC volume decreases with age.[32] In addition, the arrest of erythropoiesis by hypertransfusion was used to enrich blood with naturally aged RBC that could be characterized.[33,34] RBC from such treated rats and mice showed alterations in membrane components such as changes in the band 4.1a to 4.1b ratio and decreased cell volume, but they were not used to elucidate the mechanism of EPC.

In numerous diseases, including hemoglobinopathies, Gaucher's disease, parasite infections, diabetes mellitus and chronic kidney disease the lifespan of RBC is decreased.[13,28,35-37] RBC from subjects with such diseases have been used for EPC studies.[28] Although the results using damaged RBC provided some insight into the process of EPC *in vitro*, the use of physiologically aged RBC to elucidate the normal mechanism of EPC is a prerequisite to understand phagocytosis of sRBC and the role of this process in pathology.

In the present study, we compared the ability of macrophages to phagocytose sRBC *in vivo* and *in vitro*. An optimized protocol for mouse hypertransfusion was utilized to enrich blood with sRBC. This procedure caused an arrest of erythropoiesis, and thereby a shift in the age distribution of the RBC population towards senescence. Analysis of markers of aging indicated that sRBC from hypertransfused (ht) mice were similar to sRBC in control, unmanipulated mice with respect to all aging parameters evaluated. Blood from ht-mice was enriched for sRBC and was avidly phagocytosed by splenic macrophages following intraperitoneal injection to mice, but failed to undergo phagocytosis by cultured macrophages of splenic or bone marrow origin. The results suggest that phagocytosis of sRBC depends on an intricate cooperation between RBC aging parameters and the physiological macrophage environment.

Design and Methods

Animals

All mice were of a C57bl/6J background. All mouse experiments were approved by the Technion Animal Ethics Committee, Haifa, Israel.

Hypertransfusion

Blood from the caudal vena cava of five mice was collected into EDTA vials and 0.8 mL of this blood was immediately injected intra-peritoneally into each of five recipient mice of the same strain and sex. Two weeks later, one of these mice was transfused with the blood of two recipient mice (about 1.6 mL), and 1 day later, with blood from two more recipient mice (Figure 1A). Blood samples were drawn and analyzed on indicated days from ht- and control mice. Hypertransfusion-induced polycythemia was confirmed by evaluation of the hematocrit and the inhibition of erythropoiesis was determined by reticulocyte count.[38]

Red blood cell biotinylation and analysis of red blood cell age

In vivo biotinylation of blood cells was achieved by tail vein injection of 3 mg EZ-linked sulfo-NHS-biotin (Pierce, Rockford, IL, USA) dissolved in saline, 2 days after the first blood transfusion. On the indicated days, blood was drawn into EDTA tubes and incubated for 1 h with streptavidin conjugated to either phycoerythrin (eBioscience, San Diego, CA, USA) or fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS). The RBC were washed four times with PBS and stained for the markers mentioned below. All incubations were carried out at 37°C in the dark.

External phosphatidylserine

RBC were suspended in calcium-binding buffer and stained with annexin-V (SouthernBiotech, Birmingham, AL, USA) for 15 min following the manufacturer's instructions.

Surface CD47 levels

RBC were incubated for 30 min with rat anti-CD47 monoclonal

antibody (Santa Cruz, Santa Cruz, CA, USA), washed and then stained for 30 min with Alexa Fluor 488 chicken anti-rat antibodies (Invitrogen, Carlsbad, CA, USA).

Esterase activity

RBC were stained with 2 μM calcein-aceto-methyl ester (calcein-AM, Sigma-Aldrich), followed by incubation for 1 h with 100 μmol/L of the iron-chelator deferiprone (L1) (Apotex, Weston, ON, Canada). Esterase activity was calculated based on the calcein fluorescence of L1-treated RBC.

Levels of reactive oxygen species

RBC were stained with 0.1 mM 2′–7′-dichlorofluorescin diacetate (DCF-DA, Sigma-Aldrich) for 15 min or with 7 mM rhodamine 123 (Sigma-Aldrich) for 30 min as previously described.[39]

Flow cytometry

RBC were analyzed with a FACS-Calibur® flow cytometer (Becton-Dickinson, Immunofluorometry Systems, Mountain View, CA, USA). Twenty thousand RBC were analyzed in each sample. Markers of aging were determined separately in young RBC and sRBC populations by gating on non-biotinylated or biotinylated RBC populations, respectively. The geometric mean fluorescence intensity of each population was calculated using CellQuest Pro® software (Becton-Dickinson, Franklin Lakes, NJ, USA). For PS/cell quantification 100,000 cells were acquired.

Statistical analysis

Means and standard deviations were calculated in Excel (Microsoft Office 2010). FACS results were statistically analyzed using a two-tailed paired t-test, assuming equal variances (*http://www.physics.csbsju.edu/stats/Paired_t-test_NROW_form.html*).

Statistical analyses were done on the mean fluorescence raw data, but relative values are presented in some Figures. For CD47 relative data were analyzed with the two-tailed Student's t-test (*http://www.physics.csbsju.edu/stats/t-test_NROW_form.html*). Differences were presumed to be statistically significant when the *P* value was <0.05. *P* values <0.05 and <0.01 are marked * and **, respectively.

In vivo *erythrophagocytosis*

RBC from ht- and control-mice were washed with Alsever's solution (Biological Industries, Beit Hemek, Israel) and stained with PKH26 (Sigma Aldrich) according to the manufacturer's protocol. Labeled cells were injected to mice intraperitoneally. Four days after injection, spleens were prepared for microscopy as described elsewhere,[18] with slight modifications. Following perfusion, spleens were washed and incubated at 4°C in 30% sucrose in PBS until they sank, when they were washed again and incubated in 30% sucrose in 1:1, dH_2O:'optimal cutting temperature' compound (Sakura Finetek, Zoeterwoude, NL) at 4°C until they sank. Twelve-micron sections were visualized with a fluorescence microscope (Nikon eclipse 55i microscope, using a X-cite series 120 microscope light source system) and arbitrary areas were chosen for quantification using ImageJ software (*http://rsbweb.nih.gov/ij/*). The intensity of pixels in multiple areas was quantified and the average and standard deviation were calculated.[40] The areas of the white pulp and the red pulp were determined using bright-field micrographs. Within the red pulp, the peripheral red pulp was determined on the fluorescent micrograph as the area bordering the white pulp marginal zone. The central red pulp was the majority of the red pulp that was not in close vicinity to the white pulp.

In vitro *erythrophagocytosis*

RBC used for *in vitro* EPC were either from ht- or control-mice that did not undergo biotinylation. In addition, control RBC were treated with tert-butyl hydroperoxide as described elsewhere[30] or PS was externalized by treatment with calcium and the calcium-ionophore A23187 as also described previously.[20] Alternatively,

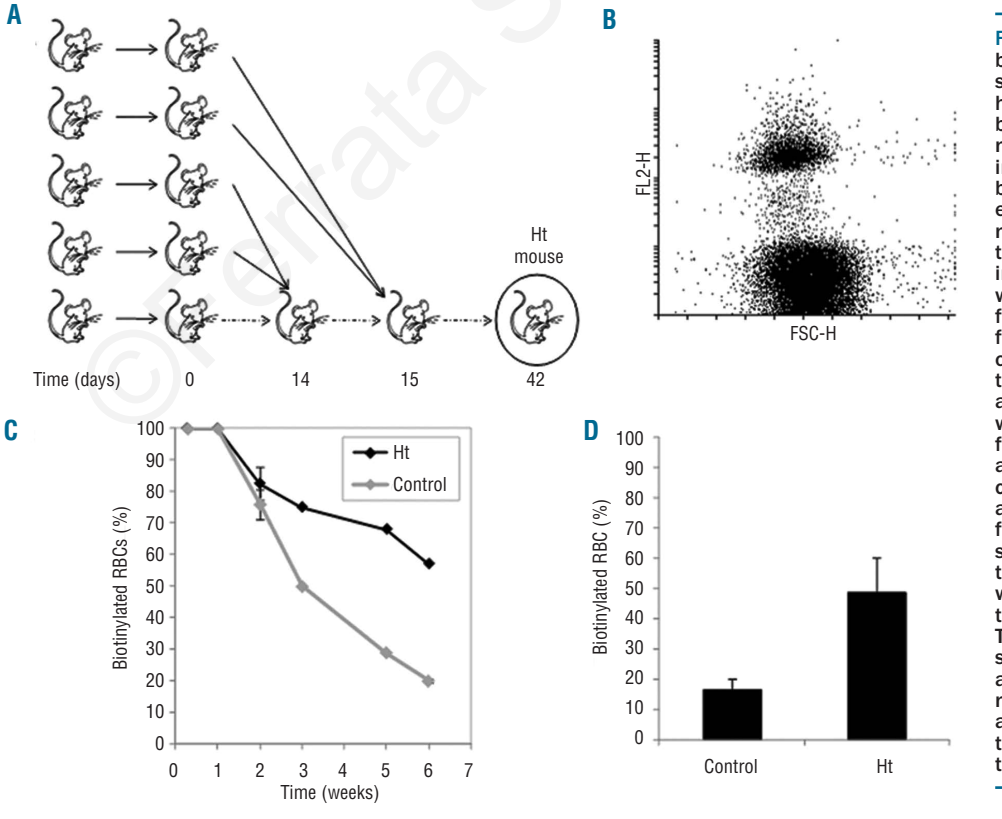

Figure 1. Enrichment of sRBCs by hypertransfusion. (A) A schematic presentation of the ht protocol. Five mice received blood from age- and sex-matched mice by peritoneal injection. Two weeks later, blood from two mice was injected into one of the transfused mice and a day later blood from two more mice was injected into the same mouse. Four weeks later, the hypertransfused (ht) mouse was used for further analyses. (B) Blood cells of a non-hypertransfused control mouse were biotinylated and after 6 weeks the blood was labeled with streptavidin-fluorescein isothiocyanate and analyzed by FACS (C) Blood-cells were biotinylated 2 days after the first transfusion in all five mice of the hypertransfusion group. Blood cells of mice that were not hypertransfused were biotinylated at the same time and served as a control. The percentage of biotinylated sRBC was determined at several time-points on a single mouse pair (C) and 6 weeks after the initiation of hypertransfusions (ht mice: n=4, control mice: n=5, *P*<0.001) (D).

RBC were opsonized as described by Abramoff *et al.*[41] In short, RBC were diluted to 1×10^9 cells/mL, incubated with 0.2 mg/mL rabbit anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) for 20 min at 37°C and washed three times with Alsever's solution.

Primary cultures of bone marrow-derived macrophages were prepared as previously described.[42] Primary cultures of splenic macrophages were prepared as described, with slight modifications.[43] Spleens were perfused with PBS with Ca^{2+} containing collagenase (100 U/mL) and DNAse (450 U/mL) (PBS-CaCD) (all from Sigma, St. Louis, MO, USA). Following perfusion, spleens were cut and incubated in PBS-CaCD for 45 min. RBC were lysed with a hypotonic buffer (17mM Tris, pH 7.6, supplemented with 140 mM NH_4Cl) for 5 min. at 37°C. The cells were then filtered, washed and cultured as previously described[43] at a concentration of 1×10^6 cells/mL. Non-adherent cells were removed after 3 days. Splenic macrophages were cultured for 1-2 weeks before use.

For *in vitro* EPC evaluation, RBC were washed with Alsever's solution and added to the macrophages (grown on coverslips) at a ratio of 10:1 (RBC: macrophages). Plates were centrifuged for 5 min at 2,600 x g and incubated at 37°C for either 30 min (splenic macrophages) or 90 min (bone marrow-derived macrophages). Non-phagocytosed RBC were lysed with hypotonic buffer. Macrophages were washed with PBS and stained with benzidine and Giemsa as previously described.[44] Cells were visualized with a light microscope (Nikon microscope eclipse 55i) and macrophages that phagocytosed at least one RBC were counted.

Results

Enrichment of senescent red blood cells by hypertransfusion

Mouse RBC are removed from the circulation after about 45 days.[45] To enrich the peripheral blood with sRBC, erythropoiesis was inhibited by an optimized hypertransfusion protocol described in the *Design and Methods* section (Figure 1A). An elevated hematocrit in the ht mice indicated that peritoneally injected RBC reached the bloodstream; the hematocrit was measured and peaked 4 weeks after the initial hypertransfusion at 80% (2 weeks after the second transfusion) (S1). When mice were sacrificed 6 weeks after the beginning of transfusions, the hematocrit was still significantly increased in ht-mice (62 ±3%) compared to in control-mice (53±2%) (n=4, $P<0.01$).

An excess of blood (polycythemia) inhibits erythropoietin production[46,47] and we detected a decrease in erythropoietin mRNA levels by semiquantitative polymerase chain reaction in the kidneys of mice 2 weeks after hypertransfusion of 3.2 mL blood (blood from 4 mice) compared to the levels in controls (S2). To evaluate the inhibition of erythropoiesis, reticulocytes were counted at different times and demonstrated a continuous decrease for the first 4 weeks and stayed low for the last 2 weeks (S3).

Age progression in RBC was evaluated with biotin bound to RBC at the beginning of the hypertransfusion protocol. After 6 weeks, and at a few time-points in between, blood was drawn and the RBC were labeled with fluorochrome-conjugated streptavidin to identify the biotinylated cells (Figures 1B, C and D). Using flow cytometry, the RBC were gated into "young" (biotin-negative) RBC or "senescent" (biotin-positive) RBC (Figure 1B). Following hypertransfusion, the RBC population was gradually enriched for biotinylated RBC compared to the blood of a control mouse (Figure 1C). Six weeks after the initiation of the hypertransfusion protocol, 49%±12 of the RBC in the ht mice (n=4) were biotinylated compared to 14%±6 in the control mice (n=5, $P<0.001$, Figure 1D). As the age of RBC at the time of their biotinylation was heterogeneous, at the endpoint of 6 weeks, biotinylated sRBC were at least 6 weeks old, but it can be assumed that some of the biotinylated cells were even older.

Analysis of aging parameters of red blood cells

The fact that in both the ht- and control-mice there was a population of biotinylated senescent and non-biotinylated young RBC made it possible to analyze aging parameters on these subpopulations within the same blood sample by fluorescent double labeling and FACS analysis. Four different aging markers were analyzed by this method 6 weeks after the initiation of the hypertransfusion protocol: externalized PS, surface CD47, cytosolic esterase activity and ROS levels. We first characterized the aging markers on biotinylated (senescent) RBC in both the ht and control blood and then compared the aging markers on sRBC from ht-mice to those on sRBC of control-mice, to substantiate our premise that the process of hypertransfusion does not affect the aging characteristics of the RBC.

Evaluation of externalized PS by annexin V showed that in blood from both ht- and control-mice, externalized PS levels were elevated to the same extent on sRBC (Figure 2A). Using a quantitative, novel two-step FACS procedure for measuring PS in aqueous solution,[48] we found nearly 5-fold more cell-free PS in the serum of ht-mice than in that of control mice (Figure 2B). Since sRBC have a higher content of surface PS, we assumed that they shed PS to a larger extent than young RBC[48] and, therefore, that the elevated serum PS in ht-mice reflected the increase in sRBC in those mice (Figure 2B).

Decreased surface CD47 is a well-accepted signal of aging[6] and we, therefore, analyzed this parameter on the RBC from ht- and control-mice. A slight, but statistically significant, decrease of CD47 surface levels was detected in sRBC from both blood sources (Figure 2C) but again no difference was detected between sRBC from ht- and control-blood. Another marker of aging, cytosolic esterase activity, was evaluated by the ability of RBC to hydrolyze the non-fluorescent and hydrophobic calcein-AM (which crosses cell membranes freely) to the polar and fluorescent form of calcein which is trapped inside the cells.[5,49] Since binding of intracellular labile iron and other transitional metals to calcein quenches its fluorescence,[50] esterase activity was measured in the presence of the iron chelator L1 that binds iron with a higher affinity than calcein. The results showed that esterase activity of sRBC was significantly lower than that of young RBC, but there was no difference between sRBC of ht- and control-mice (Figure 2D).

Since RBC senescence was reported to be associated with changes in oxidative status,[51] we evaluated ROS levels in sRBC from ht- and control-mice. Staining with two probes, DCF and rhodamine123, demonstrated lower ROS in sRBC of both the ht- and control-mice than in young RBC but no difference between sRBC of ht- and control- mice (Figures 3A and B). This finding further supports the similarity between the ht- and control sRBC populations.

Senescent red blood cells are phagocytosed by macrophages in vivo *but not* in vitro

Confirming the similarity of sRBC from ht- and control-mice with respect to their markers of aging, we next evaluated their susceptibility to undergo phagocytosis by macrophages both *in vivo* and *in vitro*. Ht- and control-RBC were labeled with the fluorescent dye PKH26 and injected peritoneally into age- and sex-matched mice. Four days later the spleens of the injected mice were analyzed for EPC. The results indicated that phagocytosis of RBC from ht-mice was higher than that of RBC from control-mice (Figures 4A-C). Blood enters the spleen at the white pulp and proceeds through the white pulp marginal zone to the red pulp from where it exits the spleen.[52] We noticed that in both ht-mice (Figures 4D-F) and control-mice (*data not shown*), EPC took place in the red pulp mainly at its periphery, adjacent to the white pulp (Figure 4E, arrows). EPC was elevated more than 2-fold in the peripheral red pulp in mice that received ht blood compared to mice that received control blood (Figure 4C).

EPC was also studied *in vitro*, comparing the phagocytosis of sRBC and damaged RBC. To evaluate the difference between physiologically aged and damaged RBC, surface PS was quantified with fluorescent annexin V on RBC treated with calcium and the calcium ionophore A23187, a treatment that induces PS externalization.[20] Much higher mean fluorescence was measured on ionophore-treated RBC than on non-treated RBC (76±21 and 29±13, respectively, n=3, $P<0.05$, Figure 5A). Control blood contained about 14% RBC that were more than 6 weeks old (Figure 1D) and these sRBC exhibited high levels of externalized PS (Figure 2A). Our results indicate that PS externalization on chemically treated RBC can reach much higher levels than PS externalization on RBC in untreated mice, including the physiologically aged RBC in that population.

To evaluate phagocytosis of control blood and sRBC-enriched blood from ht-mice, RBC were incubated with bone marrow- or spleen-derived cultured macrophages. Quantification of EPC indicated that cultured macrophages from both sources failed to phagocytose RBC from either the ht- or control-mice (Figure 5B). To verify that spleen- and bone marrow-derived macrophages in culture maintained their EPC ability, RBC from control mice were chemically damaged to externalize PS. Both bone marrow- and spleen-derived macrophages phagocytosed these RBC (Figure 5B). Bone marrow-derived macrophages also phagocytosed RBC oxidatively damaged by the oxidant tert-butyl hydroperoxide or opsonized with rabbit anti mouse IgG (Figure 5B). This suggests that damaged RBC are phagocytosed *in vitro* by macrophage primary cultures while some, yet unidentified factors essential for physiological EPC of sRBC are missing in the *in vitro* system.

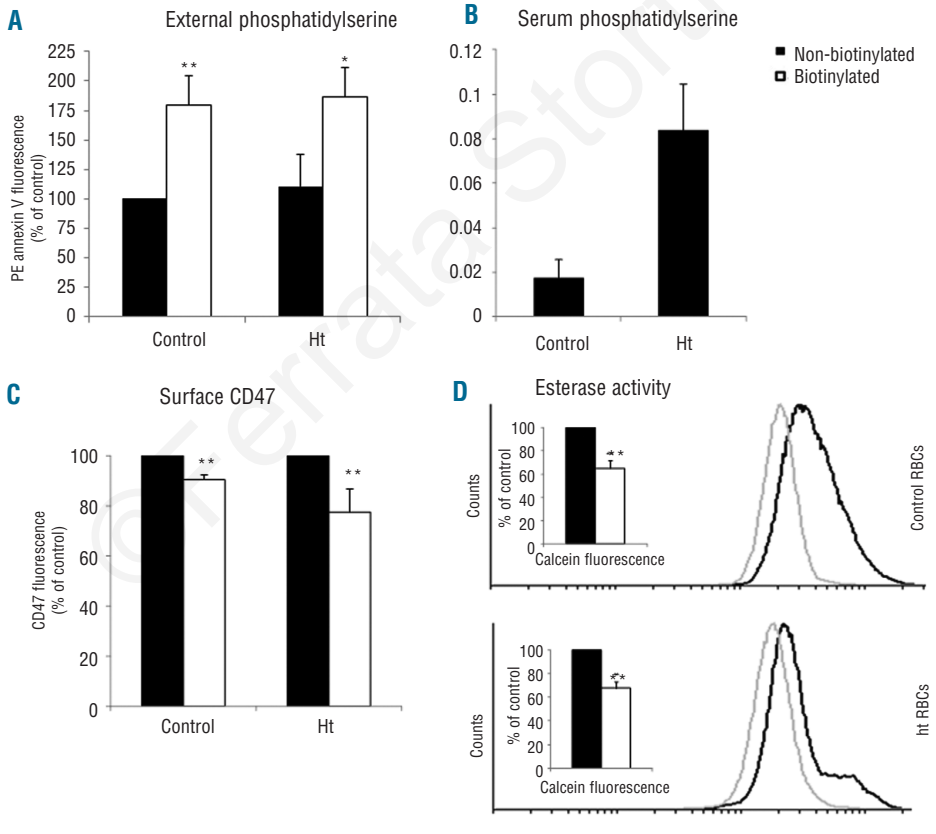

Figure 2. Markers of aging on sRBC of ht-mice are identical to aging markers on control mice. Six weeks after biotinylation, RBC were stained for biotin by streptavidin and for phosphatidylserine (PS) by phycoerythrin-annexin V (A), for CD47 by anti-CD47 antibody (C) or analyzed for esterase activity (D). Using flow cytometry, RBC were gated into non-biotinylated and biotinylated populations. The expression of each marker was determined separately in each population. (A) Externalized PS was detected with annexin V and was higher on sRBC than on young RBC in both ht- and control-mice (n=4). Results are normalized to non-biotinylated (young) RBC, (taken as 100%). (B) Serum PS measured with the two-step FACS procedure was nearly 5-fold higher in the serum of ht-mice than in the serum of control-mice (n=5). (C) Surface CD47 was detected with anti-CD47 antibodies and was significantly lower in sRBC than in young RBC of both the ht- and the control-mice (n=3). Results are normalized to non-biotinylated (young) RBC (taken as 100%). (D) Esterase activity was measured by incubation of RBC with calcein-AM (2 μM) followed by incubation for 1 h with 100 μmol/L deferiprone (L1). Esterase activity was calculated based on the calcein fluorescence of L1-treated RBC. Senescent RBC showed 40-50% lower esterase activity compared to young RBC from the same blood sample. The results of one representative experiment are represented by the histogram and the mean of three independent experiments is shown in the insert. In (A) (C) and (D) -insert, young and sRBC were compared within each group (blood from control or ht mice) with a two-tailed paired t-test (*$P<0.05$, **$P<0.01$). Biotinylated sRBC were compared between ht- and control-mice and were found not to be significantly different.

Discussion

To enrich normal blood with sRBC, we used a modified procedure of serial peritoneal blood transfusions that has been previously established in rats and mice.[33,34] In the original protocol[33,34] about 50 mice were used for serial transfusions to yield one animal enriched for sRBC. In our optimized protocol, we used only 10 mice for the same purpose. The route of peritoneally injected RBC to the blood stream has not been described in mice. In peritoneally transfused dogs, RBC enter lymph spaces, mainly in the diaphragm, from where they enter lymphatic vessels, pass the lymph nodes and reach the blood stream at the thoracic duct.[53]

The hypertransfusion protocol led to an increased hematocrit which inhibited the production of the erythroid hormone erythropoietin. Consequently, the production of new RBC was diminished and the normal age-distribution of the RBC population was shifted towards a higher proportion of sRBC (Figure 1C). We compared several properties of sRBC in blood from ht- and control-mice. To do this, RBC were labeled *in vivo* with EZ-linked sulfo-NHS-biotin and analyzed 6 weeks later. Upon analysis by flow cytometry, biotinylated and non-biotinylated RBC were detected in the same blood sample; the former reflected sRBC while the latter reflected young RBC. Each population was analyzed for markers of aging: externalized PS,[20,45,54,55] surface CD47,[6] cytosolic esterase activity[5] and ROS.[39,56] Blood from ht-mice contained more RBC with senescence markers (Figures 2 and 3), but sRBC from ht-mice did not differ from sRBC from control mice with respect to the extent of expression of these signals in sRBC. This indicated that sRBC which were enriched in the blood of the ht-mice acquired their aging signals in a physiological way by circulating in the blood stream for a period of 6 weeks or more.

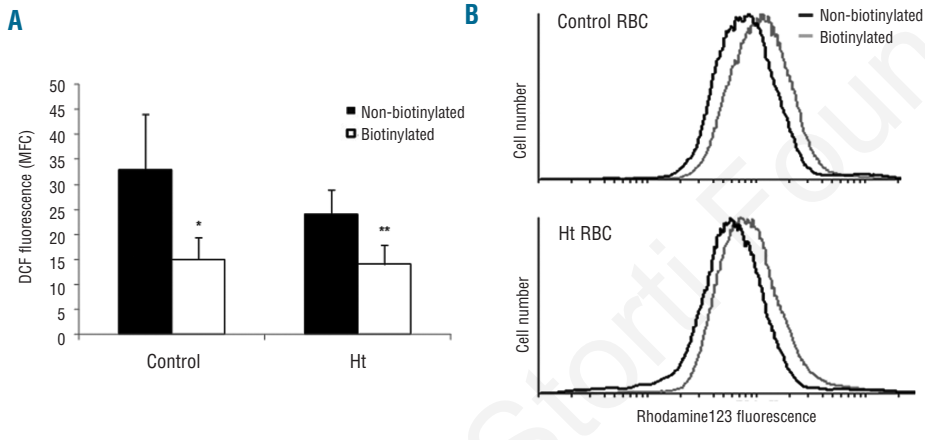

Figure 3. Reactive oxygen species (ROS) in sRBC from control and ht-mice. RBC were stained for biotin by streptavidin and for ROS by either (A) dichlorofluorescein (DCF) (n=5), or (B) rhodamine 123 (n=2). For DCF, young and sRBC were compared within each group (blood from ht- or control-mice) by the two-tailed paired t-test *P<0.05, **P<0.01. Biotinylated sRBC were compared between ht- and control-mice and were found not to be significantly different.

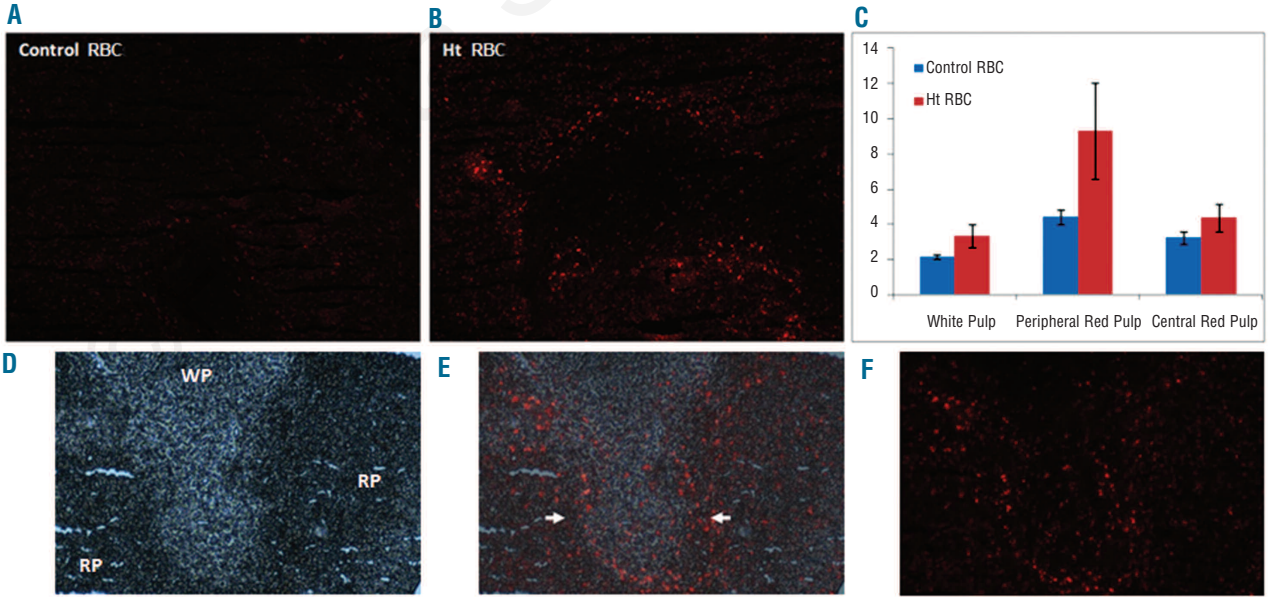

Figure 4. *In vivo* phagocytosis of sRBC. RBC of normal and ht-mice were stained with PKH26 and injected intraperitoneally into mice of the same age and sex. After 4 days, mice were perfused with heparin-PBS to remove non-phagocytosed RBC. The spleens were then removed, washed and phagocytosed RBC visualized with a fluorescence microscope (A and B). Quantification of PKH26 staining of spleen sections indicated that erythrophagocytosis was higher following injection of RBC from ht-mice than after injection of RBC from control-mice (C). The phagocytosed RBC were localized mainly in the peripheral red pulp area closest to the white pulp. (D) Bright field, white pulp (WP), red pulp (RP) (F) PKH26 fluorescence, and (E) merged, arrows pointing to peripheral red pulp.

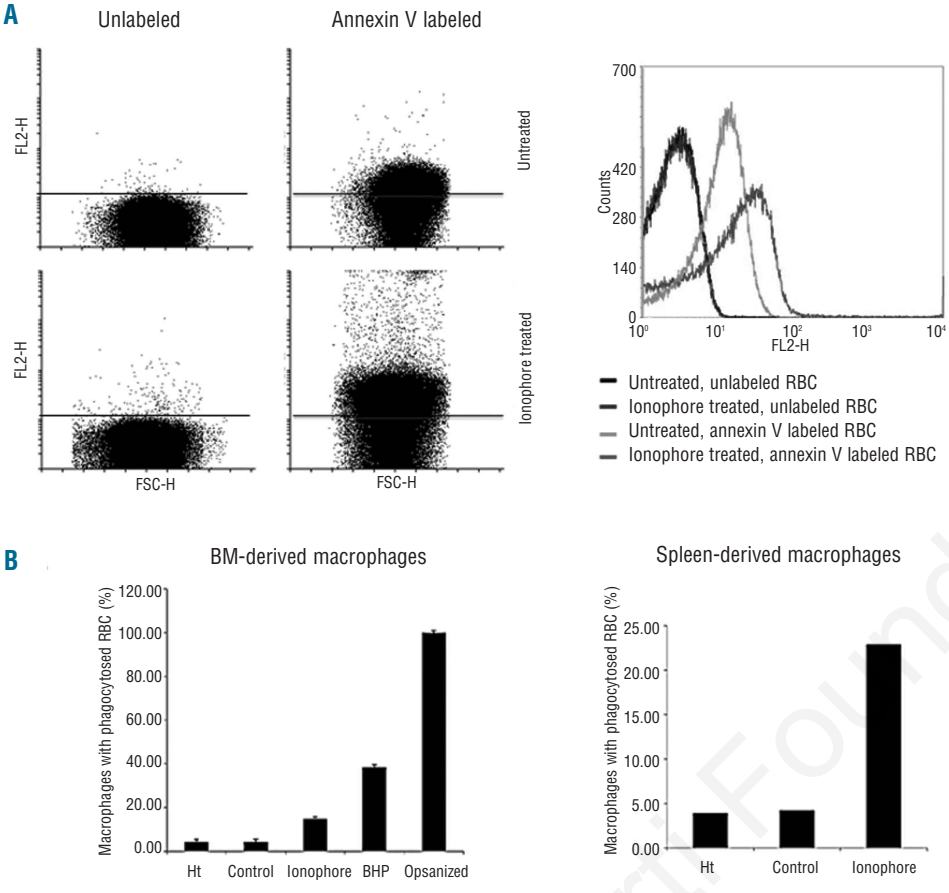

Figure 5. ***In vitro*** **phagocytosis of damaged and senescent RBC. (A) Untreated RBC and RBC treated with calcium and the calcium ionophore A23187 were compared with respect to externalized PS/RBC by labeling with fluorescent annexin V. The upper panel shows fluorescence (FL2-H)** ***versus*** **forward light scatter (FSC-H) dot plots. A cursor line was set based on annexin V-unlabeled RBC. The lower panel shows the FL2-H distribution histograms of the indicated populations. One representative experiment out of three is shown. (B) Bone marrow (BM)-derived macrophages and (C) spleen-derived macrophages were incubated for 90 or 30 min, respectively, with RBC from ht- or control-mice or with RBC treated with calcium-ionophore, BHP or opsonized with rabbit anti mouse IgG. Non-phagocytosed RBC were lysed with a hypotonic buffer. Cell cultures were stained with benzidine and the percentage of macrophages that phagocytosed one or more RBC was determined (200-700 cells were counted).**

In most cells the main origin of ROS is from mitochondrial respiration, but in RBC, ROS are mainly generated during glycolysis and by auto-oxidation of hemoglobin to methemoglobin.[57] Methemoglobin is enzymatically reduced to hemoglobin[58] and its recycling, therefore, depends on optimal enzymatic function. In aged RBC, many enzymatic activities, including methemoglobin reductase,[59] glycolytic enzymes such as hexokinase,[60] as well as the hemoglobin content are diminished, which could lead to a decrease in the rate of endogenous ROS generation. Using two probes, DCF and rhodamine 123, we demonstrated decreased endogenous ROS generation in sRBC from both ht-mice and control-mice (Figure 3B). The damage from ROS, however, accumulates and it is, therefore, higher in sRBC than in young RBC. It has previously been shown that oxidative stress is one of the cellular parameters that induce PS externalization[61] and that it is increased in sRBC.[51,62] Thus, our observation that the externalization of PS is increased may reflect cumulative increased oxidative stress (Figure 2). Taken together, the ROS generation rate was equally decreased in sRBC from ht-mice and control-mice and the evaluation of other markers of aging also showed that sRBC from ht-mice were comparable to sRBC from control mice. This demonstrates that ht-sRBC changes resulted from a physiological aging process, which was not affected by the hypertransfusion procedure. This protocol is, therefore, a legitimate procedure for enriching blood with sRBC and for better studying the properties and mechanisms of senescence and EPC.

We determined the ability of sRBC from ht- and control-mice to undergo EPC *in vivo* and *in vitro*. Following intraperitoneal injection, PKH26-labeled RBC were tracked in the spleen. More EPC was detected in spleens of animals that had been injected with RBC derived from ht-mice compared to those injected with RBC derived from control-mice (Figures 4A-C). In both cases, the highest EPC was found in the peripheral red pulp (Figures 4C-F). Arterial blood enters the spleen at the white pulp;[52] about 90% of the blood is shunted directly to the venous system and 10% is filtered through an open system that lacks endothelial lining cells. The RBC cross the marginal zone and move through the peripheral red pulp to reach the central red pulp, where they squeeze through a fenestrated endothelial wall to enter into splenic venous sinuses which connect to the peripheral circulation.[52,63] We showed that EPC levels are highest in the peripheral red pulp, suggesting that the first red pulp macrophages that interact with RBC phagocytose most of the sRBC (Figures 4C-F). Since the red pulp also functions as a RBC reservoir,[64] the removal of sRBC at entry to the red pulp ensures that the high quality RBC are stored in more central regions.

Our results of elevated EPC of ht-blood, following injection of equal amounts of fluorescently labeled RBC from ht-mice or control-mice (Figures 4A-C) support the theory of selective removal of sRBC. This, however, does not rule out a certain degree of randomness.[65] Random EPC has been reported for RBC that have either accumulated a minimal senescence signaling[66,67] or in pathological situa-

tions such as hemoglobinopathies.[65] The degree of senescence that RBC have to reach to become subject to random destruction may vary between species; using a two-step biotinylation method, it was determined that in mice some random removal exists when RBC are older than 15 days of age.[67]

We also evaluated EPC *in vitro* by incubating RBC from ht-mice or control-mice with bone marrow- or spleen-derived macrophages. The results (Figure 5B) showed that both kinds of cultured macrophages failed to phagocytose sRBC from either ht- or control-mice. However, in agreement with previous studies,[8,17,20,68] cultured macrophages phagocytosed RBC that have been used to provide a model for sRBC through treatments that induce overexpression of a single senescence property. Thus, while naturally aged RBC exposing moderate PS levels were not phagocytosed *in vitro*, substantial induction of PS externalization by treatment of control RBC with a calcium-ionophore led to significant EPC *in vitro* (Figures 2A and 5A). The moderate decrease of CD47 in naturally aged RBC (Figure 2C) was insufficient to induce EPC *in vitro*, but targeted deletion of CD47 did induce EPC *in vitro*.[6] Interestingly, in some mouse models, EPC could be detected both *in vivo* and *in vitro*. For example, in erythropoietin over-expressing mice, RBC co-exhibiting characteristics of young RBC and aging signals were phagocytosed mainly in the liver.[18] We suggest that physiological senescence represents the sum of a moderate accumulation of each of the individual signals and that these signals may act concomitantly to trigger EPC.

The inability of macrophages to phagocytose physiologically aged RBC *in vitro* could also be connected to the relationship of macrophages to their environment. Tissue macrophages differ from cultured macrophages in the physical conditions of their environment such as the partial oxygen pressure and blood flow pressure, as well as in their spatial positioning (marginal zone, white pulp and red pulp) and their interaction with other cells (e.g., endothelial cells of the blood vessels and other spleen resident cells).[52] In the liver, uptake of damaged RBC by liver macrophages (Kupffer cells) is strongly supported by the adjacent sinusoidal cells.[22] Sinusoidal cells scavenge RBC through Stabilin1 and 2, retaining RBC in the vicinity of the macrophages and thus facilitating their phagocytosis. This is an example on how macrophages depend on their tissue environment for efficient EPC. The ability of macrophages to perform significant EPC may be lost *in vitro* due to culture-related changes in their properties or inappropriate interaction with sRBC.

Increased EPC, which is reflected by reduced RBC lifespan, leads to chronic anemia - a common symptom in various diseases such as hereditary hemolytic anemias, but also exists in other pathologies such as diabetes and chronic kidney disease.[35,36] In addition, elevated EPC may affect macrophage functions such as cytokine secretion or their efficiency in removing pathogens and apoptotic cells. Transfusion of RBC that had been stored from a long time led to elevated EPC and an inflammatory cytokine response that was mediated by macrophages and iron overload.[69] We have demonstrated that physiological EPC of sRBC is a complex process that is mediated by an intricate interaction of physiologically aged RBC and macrophages which are supported by their natural environment.

Authorship and Disclosures

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