HEMATOPOIESIS

Reduced expression of CD47 during murine red blood cell (RBC) senescence and its role in RBC clearance from the circulation

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BACKGROUND: Almost 2 percent of murine blood red blood cells (RBCs) are destroyed each day and are replaced by fresh RBCs generated through the process of erythropoiesis. RBCs to be destroyed are phagocytosed by macrophages in the reticuloendothelial system, especially in the spleen. CD47 molecules on RBCs may regulate the susceptibility of RBC to destruction by phagocytosis because its recognition by inhibitory receptor (signal regulatory protein α) on macrophages sends a negative signal, which if sufficiently strong, may abort the phagocytic response altogether. The aim of this study was to investigate whether age-dependent changes in CD47 expression on circulating RBCs have a role in destruction of senescent RBCs by macrophages.

STUDY DESIGN AND METHODS: A two-step in vivo biotinylation method for labeling mouse RBCs in vivo was used to track the CD47 expression levels as well as the turnover of circulating RBCs of defined age groups.

RESULTS: Our results indicate that CD47 expression levels decrease on circulating RBCs throughout their life span in circulation. The oldest RBCs in circulation have 30 percent lower mean expression of CD47 than the youngest RBCs. Depletion of macrophages by administration of clodronate-loaded liposomes resulted in a significant decrease in the mean expression of CD47 on RBCs of all age groups and a significant accumulation of senescent RBCs in blood and spleen. A decrease in mean expression of CD47 and accumulation of senescent RBCs in macrophage-depleted mice were significantly higher for spleen RBCs compared to blood RBCs.

CONCLUSIONS: Our results provide supportive evidence for a role of decreasing CD47 expression on aging circulating RBCs in their destruction by macrophages.

The life span of circulating human and murine red blood cells (RBCs) has been estimated to be 120 and 50 days, respectively,[1-4] indicating that roughly 1 and 2 percent of circulating RBCs are destroyed each day in humans and mice, respectively. The clearance of damaged or old RBCs is mainly accomplished by the spleen macrophages.[5] The precise mechanism of recognition of damaged and old RBCs by macrophage is not clearly understood. In general it is believed that certain changes may occur in senescent RBCs that are recognized by the reticuloendothelial system, resulting in phagocytosis of these cells.[6,7] Band 3 protein modifications may take place in the RBC membrane as a result of accumulated oxidative insults and altered band 3 protein may fix naturally occurring antibody and complements.[8,9] Changes similar to apoptosis, like the extrusion of phosphatidylserine in the RBC membrane, may also occur in aged RBCs making them susceptible to phagocytosis.[10]

A role for CD47 as a marker of self on the RBCs has been proposed.[11,12] CD47 is a 50-kDa plasma membrane protein with an extracellular immunoglobulinlike domain, five transmembrane domains, and a short cytoplasmic tail. It is ubiquitously expressed on many cell types including RBCs.[13] Oldenborg and coworkers[12] showed that RBCs from CD47 knockout mice were cleared at a higher rate in wild-type mice, and the macrophages in spleen red pulp were responsible for greater clearance. Compared to control RBCs, phagocytosis of

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CD47-deficient RBCs by spleen- or bone marrow–derived macrophages was significantly higher.[12,14] CD47 on RBCs is recognized by a signal regulatory protein α or Src homology 2 domain–containing protein tyrosine phosphatase substrate-1 receptors on macrophages.[15] The interaction between the CD47 molecules on normal RBCs and signal regulatory protein α receptors on macrophages sends a negative signal to macrophages that protects RBCs from phagocytosis.[12,14] In general, it is believed that even if RBCs express determinants recognizable by macrophages as positive signal(s) to initiate the process of phagocytosis, actual phagocytosis is prevented if a sufficiently strong negative signal is simultaneously received through the recognition of CD47 on RBCs by signal regulatory protein α receptor on macrophages.[16,17] A loss of the latter interaction facilitates RBC destruction, and this mechanism has been implicated in the pathogenesis of certain types of autoimmune anemia.[17,18]

A decrease in CD47 expression on senescent RBCs has been demonstrated.[19,20] Because lower expression of CD47 would facilitate the destruction of RBCs, it is possible that a decline in RBC expression levels of CD47 molecule may be one of the mechanisms that regulate the destruction of aging RBCs. In this study, we have examined this proposition. By use of a technique that we have recently established to follow age-related changes in defined cohorts of circulating RBCs, we have studied the kinetics of decrease in CD47 expression on blood RBCs. Our results indicate that a significant decrease in CD47 expression on RBCs occurs throughout the process of aging. Systemic depletion of macrophages results in a further significant decrease in CD47 expression suggesting an accumulation of $CD47^{low}$ RBCs in circulation. A time-dependent accumulation of $CD47^{low}$ RBCs occurred in blood as well as spleens of macrophage-depleted mice, although the accumulation was greater in the spleen. Taken together, our results support a role of decreasing levels of CD47 expression on aging RBCs in the process of their removal from circulation.

MATERIALS AND METHODS

Animals

Inbred C57BL/6 mice (8-12 weeks old, 20-25 g body weight) were used throughout this study. Animals were bred and maintained in the animal house facility at JNU, New Delhi, or obtained from the National Institute of Nutrition, Hyderabad. The animals were housed in positive-pressure air-conditioned units (25°C, 50% relative humidity) and kept on a 12-hour light-dark cycle. Water and mouse chow were provided ad libitum. All the experimental protocols were approved by JNU Institutional Animal Ethics Committee and performed accordingly.

Reagents and other supplies

Sources of reagents were: biotin-X-NHS ester (Calbiochem La Jolla, CA); streptavidin allophycocyanin, rat anti-mouse CD47–fluorescein isothiocyanate (FITC), and rat anti-mouse TER119 R-phycoerythrin (BD Biosciences, San Diego, CA); anti-mouse CD16/CD32 (eBiosciences, San Diego, CA); and fetal bovine serum (FBS; HyClone Laboratories Inc., South Logan, UT). Phosphatidylcholine (LIPOID E PC) was obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Cl_2MDP (or clodronate) was a gift of Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were purchased locally and were of analytical grade. Costar (Cambridge, MA) was the source of all plastic disposable culture ware.

Macrophage depletion

Clodronate-containing liposomes were made as described earlier.[21] Mice were depleted of macrophages by the repeated intravenous (IV) injections of clodronate-containing liposomes (10 mL/kg), in the interval of 4 days, as described previously.[22] Treatment with clodronate-loaded liposomes resulted in an 80 to 90 percent depletion of macrophages in mouse spleen as adjudged by staining with F4/80 monoclonal antibodies.

Biotin labeling

In vivo biotinylation of circulating RBCs was done as described previously.[23] Briefly, mice were given three daily IV injections of 1 mg of biotin-X-NHS ester dissolved in 20 µL of dimethylformamide and 250 µL of phosphate-buffered saline (PBS). For the second biotinylation step, mice were given 0.6 mg of biotin-X-NHS ester dissolved in 12 µL of dimethylformamide and 250 µL of PBS, 5 or 25 days after the last injection of the first step, biotinylation. In the first biotinylation step, all existing RBCs were tagged with high-intensity biotin label. In the second biotinylation step, a relatively lower biotin label was imparted to RBCs generated after the first high biotinylation step. At any time point thereafter, $biotin^{high}$, $biotin^{low}$, and $biotin^{negative}$ populations of blood RBCs represented the RBCs existing before a given time point (oldest RBCs), RBCs generated within a given window of time (defined RBC population of intermediate age), and the youngest RBC population generated after the low-biotinylation step, respectively. Biotin levels on $biotin^{high}$ and $biotin^{low}$ populations of circulating RBCs are sufficiently stable in circulation to enable a clear demarcation between the three age groups of RBCs.[23]

Flow cytometry

Blood was collected in PBS containing 5 mmol per L ethylenediaminetetraacetate (EDTA). Spleen cells were

teased out from spleen in EDTA-PBS. Blood cells and spleen cells were washed three times with ice-cold normal PBS (pH 7.4) with 1 percent FBS. Before staining, spleen cells (10^6 in 50 µL) were incubated with anti-CD16/32 antibody (Fc block) for 10 minutes. Fluorescence-conjugated streptavidin or appropriate antibodies were added at concentrations recommended by the manufacturers. After incubation for 30 minutes at room temperature, cells were washed and analyzed on a flow cytometer (FACSCalibur, BD Biosciences), with computer software for acquisition and analysis (CellQuest, BD Biosciences), a minimum of 10,000 cells being analyzed for each sample. For empiric relative enumeration of CD47^{low} RBCs in blood and spleen of control and macrophage-depleted mice, RBCs expressing less than half the mean CD47 expression on control RBCs were designated as CD47^{low} RBCs.

Statistical analysis

All experiments were repeated at least three times and representative data have been presented. Statistical analysis was performed with computer software (SigmaPlot, Systat Software, Inc., San Jose, CA). Data are presented as mean ± standard deviation (SD). Significant values were calculated with a t test.

RESULTS

CD47 expression on young and old mouse RBCs in blood circulation

A depressed CD47 expression on senescent RBCs has been reported but the kinetics of age-dependent decline in CD47 expression on circulating RBCs is not known. We recently developed a new technique involving double biotinylation of circulating RBCs in mice that can be used to flow cytometrically gate on blood RBCs belonging to young, intermediate, and old age groups.[23] By use of this technique, we studied the expression of CD47 on these RBC populations.

Results in Fig. 1A show that the proportion of biotin^{high} (age, >40 days), biotin^{low} (age, 15-40 days), and biotin^{negative} (age, <15 days) RBC populations in blood were 13.3, 51.9, and 34.2 percent, respectively. Mean fluorescence intensity (MFI) of CD47 staining on these RBC populations were 176, 213, and 236, respectively, indicating that the expression of CD47 decreased with age. Forward scatter for each age group of RBCs did not show a corresponding decrease,

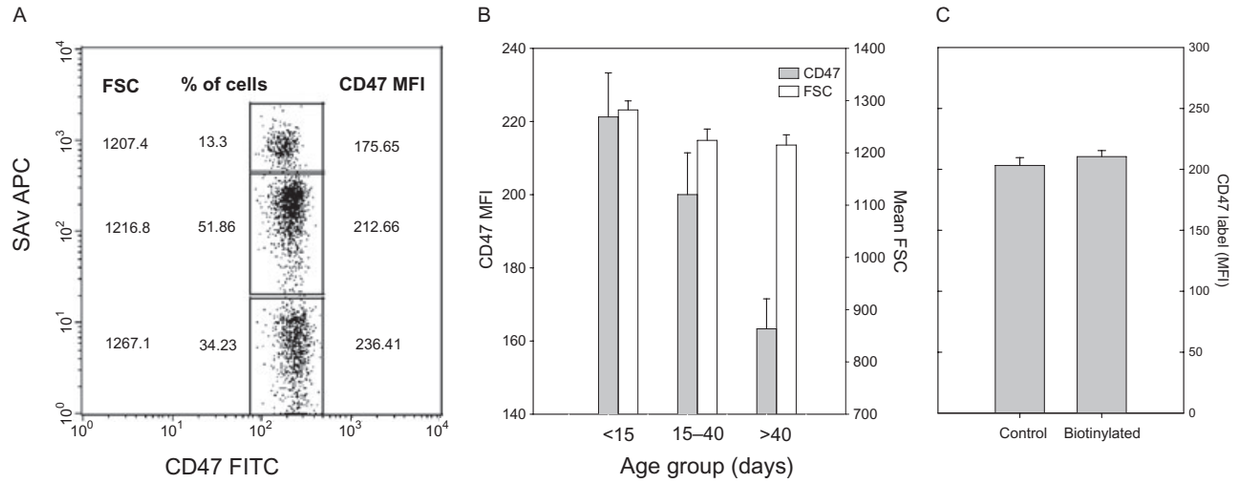

Fig. 1. CD47 expression on RBCs belonging to young, intermediate, and old age groups. Circulating RBCs were labeled by a two-step biotinylation procedure as described under Materials and Methods. The second step of low-intensity biotinylation was performed, 25 days after the last injection of the first step, high-intensity biotinylation. CD47 expression on the different age group of circulating RBCs was determined after the 15 days of the second step of biotinylation. At this time point, RBCs with high-intensity biotin (biotin^{high}) were more than 40 days of age, biotin^{low} RBCs belonged to age group 15 to 40 days, and biotin^{negative} RBCs were less than 15 days of age. RBCs were gated by staining with streptavidin allophycocyanin (SAv APC), which binds to biotin and were double-labeled with anti CD47-FITC antibody. (A) Representative flow cytometric analysis. The top-most box is for oldest RBCs, and the middle and lower boxes defines the intermediate and young age groups of RBCs, respectively. The percentage of cells in each age group and their mean forward scatter (FSC) values are shown on the left side of each box and the MFI of CD47 staining for each box is given on the right side. (B) Mean CD47 (■) expression and FSC (□) ± SD for results obtained with six mice (*p < 0.001). (C) The CD47 expression on control and in vivo biotinylated RBCs (biotin expression, >99%; MFI of biotin label, 505.6 ± 39). (B and C) Results obtained from five to six mice in each group.

indicating that lower CD47 expression on aged population was not due to decreased cell size.

Based on data from six individual mice, the mean CD47 expression in the intermediate and the old populations of RBCs was significantly lower than the CD47 expression on young RBCs ($p < 0.001$; Fig. 1B). The mean forward scatter values did not show a corresponding decrease with age (Fig. 1B).

It was important to ascertain that biotinylation itself had no effect on the binding of CD47 antibodies to RBCs. To examine this possibility, CD47 expression was compared on RBC preparations obtained from control mice and biotinylated mice immediately after biotinylation by three injections of biotin-X-NHS. More than 99 percent of RBCs from biotin-X-NHS–administered mice were biotinylated. Yet no significant difference was found between CD47 expression on control and biotinylated RBCs (Fig. 1C).

Kinetics of decline in CD47 expression with age of circulating RBCs

The double biotinylation technique[23] permits tagging of a population of RBCs generated during a defined window of time and tracking age-related changes in this cohort of cells. By use of this technique, the kinetics of decline of CD47 expression on circulating RBCs was determined. Results in Fig. 2 clearly indicate that a decrease in CD47 expression on blood RBCs is an ongoing process that starts immediately after newly generated RBCs are released into the blood stream. On average the mean intensity of CD47 staining decreased by approximately 30 percent over a period of 50 days.

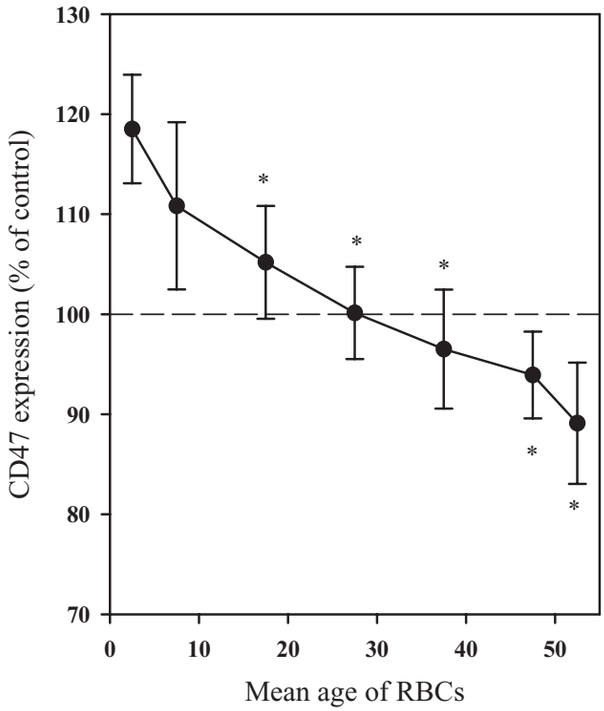

Fig. 2. Kinetics of age-dependent decline in CD47 expression on circulating RBCs. All circulating RBCs in C57BL/6 mice were biotinylated with multiple injections of the biotinylation agent as described under Materials and Methods. After 5 days, freshly generated biotin^{negative} RBCs were labeled with lower levels of biotin by a second biotinylation step with a lower dose of the biotinylation agent. Thereafter, at the indicated time points, mean CD47 expression was studied for all blood RBCs and on biotin^{low} population that represented the RBCs generated during the 5-day window between the first and second biotinylation steps. Mean CD47 expression on the age-defined population has been expressed as percentage of the mean CD47 expression on all blood RBCs. Each point represent the mean ± SD of data from five mice. *p values for significance of CD47 decline compared to the youngest RBCs (mean age, 2.5 days) ranged from 0.003 to 0.008. (●) Defined age groups of RBCs; (– – –) whole RBCs.

Effect of depletion of macrophages on CD47 expression of blood RBCs

If RBCs with relatively lower expression of CD47 marker are selectively eliminated from the circulation by macrophages, depletion of macrophages should result in accumulation of CD47^{low} RBCs in blood and consequently a decrease in mean CD47 expression on blood RBCs. This proposition was examined by depleting macrophages by administering to mice liposomes loaded with clodronate (dichloromethylene-bisphosphonate). Results in Fig. 3 show that the mean CD47 expression on circulating RBCs decreased by 14 percent in macrophage-depleted mice ($p < 0.005$). Because the decrease in CD47 expression is not restricted to older RBCs and begins immediately after the release of RBCs into circulation (Fig. 2), it was of interest to know whether any particular age group of RBCs contributed more to the decrease in CD47 expression in RBCs in macrophage-depleted mice. Results in Table 1 show that the extent of decline in CD47 expression was comparable in all the three age groups of RBCs from macrophage-depleted mice. Interestingly, however, there was a 64 percent increase in the proportion of oldest (>40 days of age) RBC population in macrophage-depleted mice, whereas the relative proportions of RBCs of intermediate age groups did not change and a 36 percent decrease in the proportion of youngest (<15 days) RBC population was noted (Table 1).

Effect of macrophage depletion on expression levels of CD47 on blood and spleen RBCs

The spleen is a major site of destruction of RBCs. Senescent and damaged RBCs have poor deformability, which

may lead to their entrapment in the spleen.[24] Furthermore, a weakened protective signal due to depressed CD47 expression on aged RBCs may facilitate their destruction by macrophages in spleen. In macrophage-depleted mice, therefore, an enrichment of CD47^{low} RBCs may be expected. To test this possibility, CD47 expression was examined on blood and spleen RBCs in control and macrophage-depleted mice. Because the spleen has a higher proportion of cells of lymphocyte lineage, TER119 antibody (a marker specific for erythroid cells) was used to gate on RBCs. Results in Fig. 4A show the flow cytometric histograms of CD47 expression on TER119+ blood and spleen RBCs from control and macrophage-depleted mice. A clear shift toward lower CD47 expression on RBCs from macrophage-depleted mice is observed in these histograms. A decrease in mean CD47 expression was significant for both blood- and spleen-derived RBCs from macrophage-depleted mice, but the magnitude of decrease was twice for spleen RBCs (33% decrease) than for the blood RBCs (16% decrease).

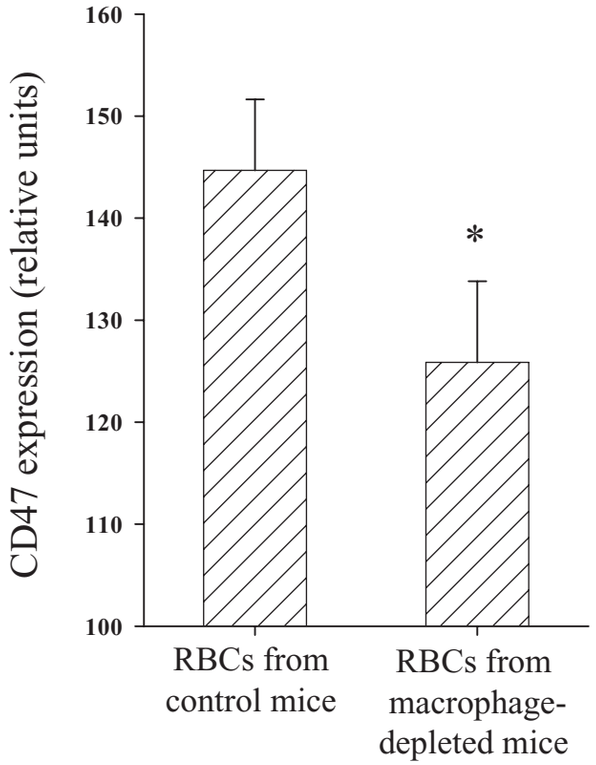

Fig. 3. Effect of macrophage depletion on the CD47 expression on blood RBCs. Mice were depleted of macrophages for a period of 7 days by repeated administration of the clodronate liposomes as described under Materials and Methods. Blood was collected from the tail vein, and CD47 expression label was determined by flow cytometry after staining with anti-mouse CD47-FITC. Each bar shows the mean ± SD of data from five mice. *p < 0.0005.

Kinetics of accumulation of CD47^{low} RBCs in macrophage-depleted mice

A relatively greater decline in mean CD47 expression on spleen RBCs from macrophage-depleted mice could have resulted from the fact that spleen is the site of destruction of RBCs where aged RBCs would tend to accumulate. To further confirm this proposition, we examined the kinetics of accumulation of CD47^{low} RBCs in blood and spleen of control and macrophage-depleted mice. For this purpose, macrophage deficiency was maintained for a period of 10 days in mice by repeated administration of clodronate liposomes and the kinetics of accumulation of CD47^{low} RBCs was examined in blood and spleen. In these experiments, mean CD47 expression was estimated for whole RBC population under study and RBCs with CD47 expression less than 50 percent of the mean CD47 expression were empirically designated as CD47^{low} RBCs. Results in Fig. 5 show that in control mice, CD47^{low} RBCs constituted 0.8 and 1.8 percent of all blood and spleen RBCs, respectively. There was a progressive accumulation of CD47^{low} RBCs in blood and spleen of macrophage-depleted mice. After 10 days of sustaining the macrophage-depleted state, the percentage of CD47^{low} RBCs had reached 4 and 13 percent in blood and spleen, respectively. These results suggest that in absence of macrophages, CD47^{low} RBCs

TABLE 1. Effect of macrophage depletion on the age distribution of blood RBCs and mean CD47 expression in different age groups of RBCs*

		Age group of RBCs (days)†		
Mice	Variable	<15	15-40	>40
Control	CD47 expression	156.88 ± 8.33	147.91 ± 6.99	124.23 ± 4.75
Macrophage-depleted	CD47 expression	133.85 ± 7.94	131.53 ± 7.35	112.99 ± 5.91
	Change (%)	–14.7[b]	–11.1[b]	–9.1[b]
Control	Percent cells	32.12 ± 2.16	48.93 ± 1.25	16.28 ± 1.89
Macrophage-depleted	Percent cells	20.40 ± 1.22	50.39 ± 2.16	26.58 ± 1.34
	Change (%)	–36.5[c]	+3.0[a]	+63.3[c]

* Biotin double-labeled blood RBC preparations from control and macrophage-depleted mice were stained with streptavidin allophycocyanin and CD47-FITC antibody. Young, intermediate, and old populations of RBCs were gated and CD47 expression on different age groups of RBCs was determined as described in legend to Fig. 1. All results are mean ± SD of data from five mice.

† Significance of differences between RBCs from control and macrophage-depleted mice: [a]p < 0.05, [b]p < 0.01, [c]p < 0.001.

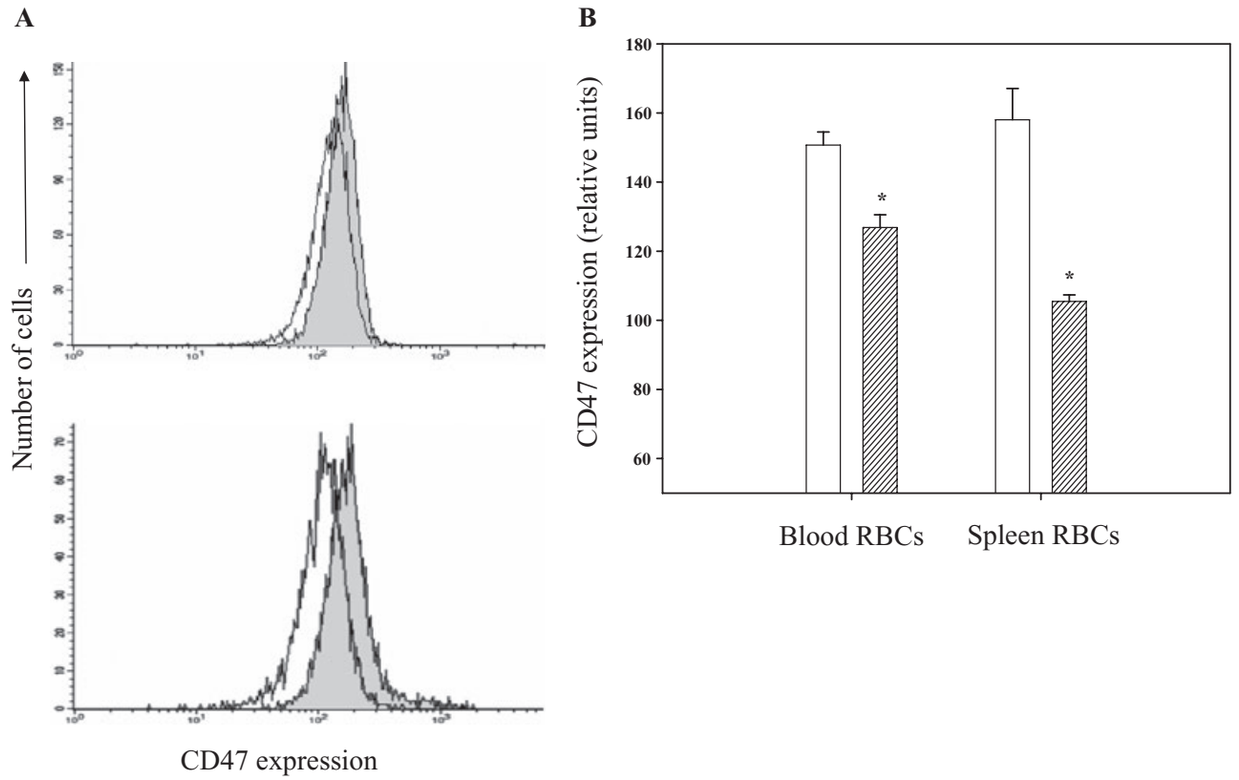

Fig. 4. CD47 expression on the blood and spleen RBCs after the depletion of macrophages. Mice were depleted of macrophages for a period of 10 days by repeated administration of clodronate-loaded liposomes as described under Materials and Methods. The CD47 expression on spleen and blood RBCs was determined by double staining with TER119-PE and CD47-FITC antibodies. (A) Representative profiles of CD47 expression on TER119+ gated RBCs populations from blood (top) and spleen (bottom) of control (■) and macrophage-depleted mice (□). A summary of data of MFIs of CD47 expression from five such experiments is given in B (□, control mice; ▨, macrophage-depleted mice). *p < 0.0001.

progressively accumulate in blood and spleen, the rate of accumulation being significantly greater in spleen.

DISCUSSION

There is a rapid turnover of RBCs in blood. Almost 1 to 2 percent of circulating RBCs are removed from circulation each day and are replaced by an equal number of fresh RBCs. Phagocytosis by macrophages in reticuloendothelial systems of spleen and liver may be the main mechanisms of RBC destruction,[4,24] although lysis as an alternative mechanism of RBC destruction has also been suggested.[25]

RBCs earmarked for destruction must express some unique markers that may activate macrophages to initiate the phagocytic process. For example, opsonization by autoantibodies that bind old RBCs and the attendant complement fixation may provide a positive signal to activate macrophages.[8,16,24] In recent years a qualitatively different type of interaction between RBCs and macrophages has also been recognized. Molecules like CD47 and sialic acid residues may interact with their corresponding receptors on macrophages and send a negative signal restraining the macrophages from initiating a phagocytic response.[16,17,26] According to the currently held view of RBC-macrophage interaction, phagocytosis of a given RBC depends on the net positive and negative signals it sends to macrophages. Thus, RBC destruction can result from enhanced positive signal as well as a decreased negative signal originating in RBCs or both. Indeed, alterations in both types of signals have been implicated in the destruction of RBCs in a variety of autoimmune hemolytic disorders as well in the physiologic turnover of RBCs.[16-18]

By use of a new double biotinylation technique to track age-related changes in circulating RBCs, we found that the CD47 expression started to decrease immediately after the cells are released into circulation and continued throughout the aging process (Fig. 2). Overall there occurred a 30 percent decrease in CD47 expression as RBCs aged in blood circulation. Our results thus suggest that the intensity of the negative signal that RBCs send to macrophages declines gradually on aging population of RBCs. To further understand whether this decrease in

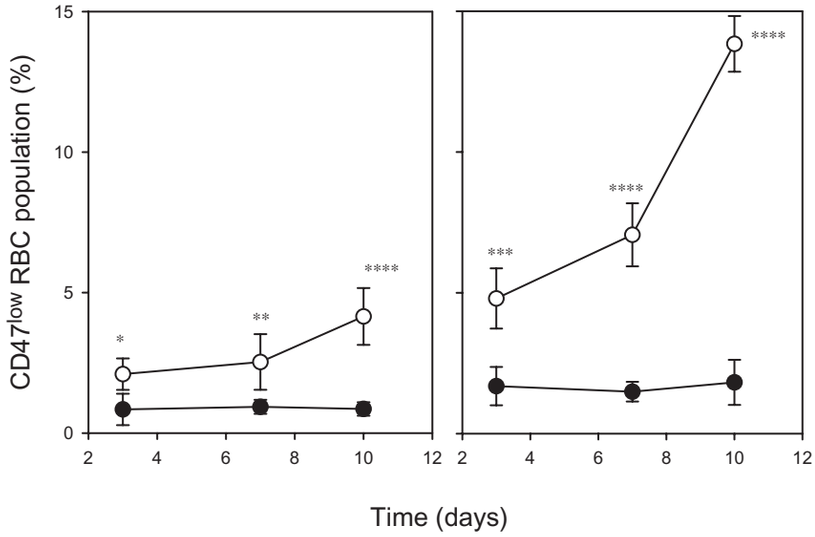

Fig. 5. Kinetics of accumulation of CD47^{low} RBC population in blood and spleen of control (●) and macrophage-depleted (○) mice. Mice were depleted of macrophages for periods of 3, 7, and 10 days by administration of clodronate-loaded liposomes as described under Materials and Methods. Blood and spleen cells were double-labeled with TER119 and anti-CD47 antibodies as described in legend to Fig. 4. CD47^{low} RBC population in the control and macrophage-depleted mice on the blood (left) and spleen (right) RBCs were enumerated from the CD47 expression histograms as the percentage of RBCs that had less than 50 percent of the mean CD47 expression of control RBCs. Each point in the graph shows the mean ± SD of data from five mice. At each time point the significance of difference between the control and macrophage-depleted group was determined. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

CD47 expression is correlated with macrophage-mediated destruction of RBCs, we depleted macrophages systemically in mice by administration of liposomes containing clodronate. Depletion of macrophages resulted in a significant decrease in the mean CD47 expression on circulating RBCs (Fig. 3) that could be due to the accumulation of CD47^{low} RBCs in blood that were earmarked for destruction. Because the decrease in CD47 expression was a continuous process, it was necessary to examine whether accumulation of CD47^{low} RBCs in macrophage-depleted mice occurred uniformly in all age groups of RBCs or was different for different age groups of RBCs. Results of these experiments indicated that the mean decrease in CD47 expression was comparable in all age groups of blood RBCs from macrophage-depleted mice. There was, however, a substantial accumulation of old RBCs in blood of macrophage-depleted mice that may result from extended survival of RBCs that would otherwise have been destroyed. We also observed a significant decrease in the proportion of young RBCs but no change in the intermediate group. Depressed erythropoietic activity as a result of macrophage depletion has been reported[27] and could have contributed to the relative decline in the relative numbers of young RBCs.

Barbe and coworkers[28] and Giuliani and coworkers[29] have shown that there is a small yet significant increase in blood RBC counts in rats and mice treated with clodronate liposomes to deplete macrophages, suggesting that in absence of the clearing mechanism, RBCs may accumulate in the system. Our results demonstrate a time-dependent accumulation of CD47^{low} RBCs occurred in macrophage-depleted mice. A substantially greater accumulation of CD47^{low} RBCs in spleen compared to blood of macrophage-depleted mice may be due to the fact that aging RBCs move to spleen for destruction. It should be noted that our definition of CD47^{low} RBCs was only empiric. We defined CD47^{low} RBCs as the ones that had less than half the mean CD47 expression on whole RBC population. This assumption was made just for relative enumeration of CD47^{low} RBCs and we got essentially similar results if we fixed the window defining CD47^{low} RBCs at 30 or 70 percent, rather than 50 percent of the mean CD47 expression (results not shown).

An important question is whether up to 30 percent decrease in CD47 expression in aging RBCs is sufficient to influence their susceptibility to phagocytosis by macrophages. Olsson and colleagues[30] have recently shown that the expression of CD47 on RBCs of heterozygous CD47+/– mice is 50 percent of the CD47 levels on homozygous CD47+/+ mice and opsonized CD47+/– RBCs were markedly more susceptible to phagocytosis by macrophages. Furthermore, a 25 percent reduction in CD47 expression on mouse RBCs induced by treatment with deoxyglucose is associated with a marked increase in rate of clearance of these cells in vivo (our unpublished results). Thus, a graded decline in CD47 expression may influence their clearance. Overall susceptibility of RBCs to macrophages is determined by the net positive and negative signals originating from RBCs. Our observations support the hypothesis that CD47 expression on RBCs may be one such important factor determining the susceptibility of aging RBCs for destruction by macrophages.

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