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 12hrs 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 Fluorescein-Isothiocyanate (SAv FITC), Streptavidin Allophycocyanin (SAv APC), Streptavidin Phycoerythrin (SAv PE), Rat anti mouse CD47 Fluorescein-Isothiocyanate (FITC), Rat anti Mouse Ter 119 R- Phycoerythrin (R-PE) (BD Biosciences San Diego, CA, USA) Anti-mouse CD147-FITC and Anti-mouse CD16/CD32, F4/80 FITC (eBiosciences San Diego, CA, USA) Thiazole Orange, Dimethylformamide (DMF), Calcium ionophore A23187, 2-deoxyglucose (DOG), Phenylhydrazine, tert-Butyl hydroperoxide (tBHP), Quinine, Valinomycin, Bovine Serum Albumin and other reagents were from Sigma-Aldrich (India). 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) and Fluo 3AM was purchased from molecular probes, Eugene, OR, USA. Fetal Bovine serum (FBS), Hyclone Laboratories Inc. (South Logan, UT), USA; Clodronate containing liposomes were obtained from Dr. Nico van Rooijen, (Department of Molecular Cell Biology, Vrije University, Amsterdam’ The Netherlands) All other chemicals were purchased locally and were of analytical grade. Costar (Cambridge, MA), USA was the source of all plastic disposable culture ware.
Materials and Methods

Biotin labeling

For the biotinylation of erythrocyte surface proteins N-hydroxysuccinimide (NHS) ester of biotin was used. This reagent condenses with the primary amines of the proteins and gets covalently attached with the protein on the erythrocyte surface. For *in vivo* biotin labeling mice were given three daily i.v. injections of 1 mg of biotin-X-NHS Ester (BXN) dissolved in 20 μl of dimethylformamide (DMF) and 250 μl of phosphate buffered saline (PBS, NaCl 8 g/lit, Na₂HPO₄·2H₂O 1.44 g/lit, KCl 0.2 g/lit, KH₂PO₄ 0.2 g/lit, pH 7.4). For the second biotinylation step, mice were given 0.6 mg of BXN dissolved in 12 μl of DMF and 250 μl of PBS, 5 or 25 or 30 days after the last injection of the first step biotinylation. The time interval in between first and second step biotinylation was determined according to the requirement of the experiment.

Macrophage depletion

Mice were depleted of macrophage by the use of clodronate loaded liposomes, prepared as per the method described before (Van Rooijen and Sanders 1994), and stored at 4°C and used within one month of preparation. For the depletion of splenic and hepatic macrophages intravenous route of injection was followed. The liposome suspension was mixed well and allowed to come at room temperature before the injections. For sustaining macrophage depleted state for longer durations mice were repeatedly injected with the clodronate loaded liposome (10ml / Kg body weight), at intervals of 4 days. Within one day after a single administration of clodronate loaded liposomes, splenic and hepatic macrophages are depleted (Van Rooijen and Sanders 1994). In our experiments mice were considered macrophage depleted after one day of the clodronate liposome administration. The efficacy of the every batch of liposome was checked by determining the macrophage population in the spleen after the administration of clodronate loaded liposomes by flow cytometry using anti F4/80 antibody that specifically stains macrophages.
Staining of cells and Flow cytometry

Mouse blood was collected in phosphate buffer saline (PBS, pH 7.4) containing 5 mM ethylenediamine tetra-acetic acid (EDTA). Spleen cells were teased out of the spleen in EDTA PBS. Blood cells and spleen cells were washed 3 times with ice cold PBS with 1% FBS. Before staining, spleen cells (10^6 in 50 µl) were incubated with anti-CD16/32 antibody (Fc block) for 10 min. Fluorescence conjugated streptavidin or appropriate antibodies were added at concentrations recommended by the manufacturers. After incubation for 30 min at room temperature (in the dark) cells were washed and analyzed on a BD FACS Calibur flow cytometer (with blue and red lasers), using Cell Quest software for acquisition and analysis. Filtered (0.22µm filter) saline was used as sheath fluid to run the samples. A minimum of 10,000 cells were analyzed for each sample. For erythrocyte study FSC, SSC and fluorescent signals were studied on the logarithmic scales. For empirical relative enumeration of CD47_{low} or CD147_{low} erythrocytes in blood and spleen of control and macrophage depleted mice, erythrocytes expressing less than half the mean CD47 or CD147 expression on control erythrocytes were designated as CD47_{low} or CD147_{low} erythrocytes respectively.

Detection of biotin labeling on the erythrocytes

To detect biotin on the erythrocytes, biotin labeled cells were stained with streptavidin conjugated with different fluorochrome (SAv FITC/SAv PE/SAv APC) in Hepes Buffered Saline (HBS, 10mM Hepes, 165mM NaCl and pH was adjusted to 7.4 with 2N NaOH) for 30 minutes at room temperature. Old, intermediate and young age erythrocytes biotinylated by the DIB technique were gated on the basis of the intensity of the fluorescence signals after staining with fluorescent conjugated streptavidin.
Materials and Methods

Determination of reticulocyte population in the circulating erythrocytes

Reticulocyte population in the blood was determined by using Thiazole orange, a dye which specifically binds to the nucleic acids. Cells were incubated with thiazole orange (50 ng/ml) in HBS for 30 min at room temperature and washed. Fluorescence signals were detected in the FII Channel after excitation by 488nm laser. The stock of thiazole orange was made at a concentration of 1mg/ml in methanol and stored at -20°C.

Determination of Macrophage population in the spleen

To detect macrophage population in spleen cells derived from control and macrophage depleted mice, spleens were teased in PBS containing 5mM EDTA and cells were washed three times with PBS containing 1% FBS. Spleen cells were stained with anti mouse F4/80 antibody conjugated with FITC. Fe block (CD16/32 antibody) was used to block Fc receptor on macrophages before staining. For flow cytometry FSC and SSC were kept on the linear scale.

Detection of erythrocyte population.

Erythrocyte population in the spleen cells were gated by staining with phycoerythrin conjugated monoclonal antibody Ter 119. Ter119 antigen is expressed on erythroid cells from pro-erythroblast through mature erythrocyte stages. Before staining with this antibody Fc receptors on the cells were blocked by incubation with Fc block (CD16/32 antibody).

Determination of Phosphatidylserine exposing erythrocytes

Phosphatidylserine expressing erythrocyte population was determined by flow cytometry after staining of the cells with fluorochrome conjugated Annexin V. Fresh blood erythrocytes or erythrocytes after various treatments were washed with HEPES buffered saline (HBS) and resuspended in the HBS containing 2.5 mM Calcium chloride. One million cells were taken and incubated with Annexin V FITC/ APC for
20 minutes at room temperature, washed and resuspended in the HBS with 2.5mM CaCl₂ and analyzed on flow cytometer immediately.

**Determination of intracellular Calcium ion**

Intracellular calcium ion concentration was estimated by using Fluo 3 dye as described before (Minta *et al* 1989). Cells were stained with Fluo 3 acetoxyethyl ester (Fluo 3 AM, final concentration 1 μM) in the HBS for 30 minutes at room temperature washed and resuspended in the same buffer. Cells were analyzed on flow cytometer.

**In vitro treatment of erythrocytes**

A suspension of 2% erythrocytes was made in 10 mM Hepes buffered saline and incubated in the presence of various reagents [Glucose, DOG, Calcium Chloride, Calcium ionophore A23187 (stock solution in DMSO), Quinine (stock solution in ethanol), Phenyl Hydrazine (PhyZ), tert-Butyl hydroperoxide (t-BHP)] in the 96 well U bottom culture plate for different times at 37°C and 5% CO₂. After harvesting the erythrocytes cells were washed three times with HBS.

**Study of in vivo clearance of phosphatidylserine exposing erythrocytes**

Mouse blood (500μl) was collected in anticoagulant Heparin (10 units) and mixed with a solution of 1.3 mg biotin X NHS (BXN) dissolved in 26 μl DMF and 4 ml of PBS and incubated at 37°C for 30 minutes with gentle shaking. After incubation erythrocytes were washed three times with PBS containing 1% FBS. A 16% suspension of biotinylated erythrocytes was prepared in the HBS with 500μM CaCl₂ and incubated for 3 minutes at 37°C. Calcium ionophore (A23187) was added to a final concentration of 1μM and cells were further incubated for 60 minutes. After treatment erythrocytes were washed once with HBS containing 2.5mM EDTA (to remove calcium) and thrice with Hepes buffered saline containing 1% bovine serum albumin (BSA, to remove the ionophore). Subsequently erythrocytes were washed twice with plain Hepes buffered saline, resuspended in HBS and injected in the mouse.
through tail vein. Blood samples were collected from the mice at different time points and erythrocytes were washed and stained with SAv PE and Annexin V FITC in the Hepes buffer saline containing 2.5mM CaCl₂.

**Study of in vivo clearance of CD47<sup>low</sup> erythrocytes**

A 5% suspension of RBC was made in PBS and erythrocytes were incubated with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, final concentration 10 µM) for 15 minutes at 37°C. (CFSE stock was made in DMSO, 5 mM and stored at -20°C). Erythrocytes were washed twice with PBS containing 1% FBS, once with plain HBS and 2% erythrocyte suspension was made in the same buffer. Erythrocytes were incubated in 6 well plate in the presence of 5 mM Glucose or 5 mM DOG for 8 hrs at 37°C. After incubation erythrocytes were harvested and washed three times with HBS, resuspended in HBS and injected in the mouse through tail vein. Blood samples were collected before and after 5, 15, 30, 60,120 and 240 minutes of infusion, from the tail vein and washed with HBS. CFSE labeled erythrocytes in the whole erythrocytes was determined by flow cytometry. For survival kinetics, the CFSE labeled erythrocytes at 5 minute time point was considered as 100 and results at different time points expressed as percentage of this control. To study the phagocytosis of CFSE stained erythrocytes spleen cells were teased out from the spleen in the EDTA PBS and washed with PBS. Spleen cells were depleted of unphagocytosed erythrocytes by the hypotonic lysis. For this purpose 500µl of water was added drop by drop to the pellet of spleen cells and the tube was vortexed for 15 seconds, followed immediately with the addition of equal volume of 2X PBS. Then cells were washed thrice with PBS and resuspended in the same buffer and immediately analyzed on a flow cytometer.

**Statistical analysis**

Statistical analysis was carried out by using Sigma plot software. Significance of differences in mean values were calculated by using students-t-Test.