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Integrin-associated protein (CD47) is a putative mediator for soluble fibrinogen interaction with human red blood cells membrane

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ABSTRACT

Fibrinogen is a multifunctional plasma protein that plays a crucial role in several biological processes. Elevated fibrinogen induces erythrocyte hyperaggregation, suggesting an interaction between this protein and red blood cells (RBCs). Several studies support the concept that fibrinogen interacts with RBC membrane and this binding, due to specific and non-specific mechanisms, may be a trigger to RBC hyperaggregation in inflammation. The main goals of our work were to prove that human RBCs are able to specifically bind soluble fibrinogen, and identify membrane molecular targets that could be involved in this process. RBCs were first isolated from blood of healthy individuals and then separated in different age fractions by discontinuous Percoll gradients. After isolation RBC samples were incubated with human soluble fibrinogen and/or with a blocking antibody against CD47 followed by fluorescence confocal microscopy, flow cytometry acquisitions and zeta potential measurements. Our data show that soluble fibrinogen interacts with the human RBC membrane in an age-dependent manner, with younger RBCs interacting more with soluble fibrinogen than the older cells. Importantly, this interaction is abrogated in the presence of a specific antibody against CD47. Our results support a specific and age-dependent interaction of soluble fibrinogen with human RBC membrane; additionally we present CD47 as a putative mediator in this process. This interaction may contribute to RBC hyperaggregation in inflammation.

1. Introduction

Fibrinogen is a multifunctional protein with 340 kDa, synthesized by hepatocytes and secreted into the bloodstream. It consists in two sets of three distinct polypeptide chains: α (610 aa), β (461 aa) and γ (411 aa). In physiological conditions, fibrinogen circulates at concentrations ranging from 2 to 4 mg/mL Fibrinogen is a component of the coagulation cascade as well as a determinant in plasma viscosity and erythrocyte aggregation [1–5]. Recently the role of fibrinogen has been expanded beyond its hemostatic and hemorheological functions. It was demonstrated to be an acute phase protein mediating various processes involved in inflammation. Several biological responses occur due to its ability to affect and bind to cells involved in the inflammatory process. For such, fibrinogen displays binding sites for unique and very specific cellular receptors expressed by these cells [3,5].

Under inflammatory conditions fibrinogen synthesis is upregulated. This increase in plasma fibrinogen levels affects hemorheological parameters such as red blood cell aggregation, blood viscosity and consequently interferes with blood flow [4,6,7]. Additionally, elevated plasma fibrinogen levels are a major independent cardiovascular risk factor [8]. This protein is also considered as a "non-specific glue" leading to increased adhesiveness/aggregation of both Red Blood Cells (RBCs) and leukocytes [7].

RBCs represent the largest group of cellular elements of blood, and therefore these cells significantly influence blood flow and its rheology. RBC deformability and aggregation are its major characteristics that modulate microvascular hemorheology [9]. RBC aggregates are responsible for excluding leukocytes from the bulk suspension inducing their interaction to the endothelium, promoting their migration and thus favoring inflammation [8]. Erythrocyte hyperaggregation occurs in diabetes, atherosclerosis, arterial hypertension, ischemic heart disease, stroke and other vascular pathologies [4,5,10,11]. Inhibition of erythrocyte aggregation can prevent and reduce the overall thromboembolic risk [12].

Since elevated fibrinogen induces RBCs hyperaggregation, this suggests that it interacts with the erythrocyte membrane. Fibrinogen Aα chain has been proposed as a binding site responsible for this interaction [13,14]. Lominadze et al. [15], using murine RBCs and ghosts, suggested that soluble fibrinogen binds to the erythrocyte

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membrane through non-specific and also specific mechanisms that could trigger RBC hyperaggregation in inflammation. They also propose that this interaction could be Arginine–Glycine–Aspartate (RGD) peptide dependent [15]. More recently, Carvalho et al. reported, in two different works [16,17], using atomic force microscopy (AFM) as main technique, the existence of a single-molecule interaction between fibrinogen and an unknown receptor on the RBC. In the first work [16], Carvalho et al.'s main conclusion was that this receptor is an $\alpha_{IIb}\beta_3$-related integrin. The second work [17] is focused in the variations on fibrinogen binding to RBC membrane during the cell aging, showing that younger RBCs bind more to fibrinogen than the older ones. This aspect will be explored in detail in our study

Erythrocyte membrane surface have several molecular targets that could act as receptor/target for soluble fibrinogen such as the integrin-associated protein (IAP) or CD47, integrin cell adhesion molecule-4 (ICAM-4), basal cell adhesion molecule (BCAM/LU), CD147, CD44, CD58, and glycosphingolipids (gangliosides). All these molecules are essential in erythropoiesis and some are also important during erythrocyte clearance from the bloodstream [18–20]. Moreover, developments in proteomic field also have made possible to perform complex analysis of membrane proteome from RBCs given us a more accurate knowledge from RBCs membrane content [21,22].

Although fibrinogen–erythrocyte interaction has been properly established, we think that there were several aspects that needed to be clarified. In summary, we report that there are not enough data to support the idea that soluble fibrinogen–human erythrocyte interaction is integrin dependent but it is undoubtedly age dependent. Additionally, we also propose CD47 as a putative mediator for this binding.

2. Materials and methods

2.1. Chemical reagents

K_3EDTA tubes from Vacuette-Greiner Bio One (Austria), NaCl (S7653), $CaCl_2.2H_20$ (22350-6), $MgSO_4.7H_20$ (63140), $MgCl_2.6H_20$ (M9272), Percoll (P-1644), Albumin (A-2153), Poly-L-lysine P-9404, Fibrinogen fraction I, type I from human plasma (F-3879), Arginine–Glycine–Aspartate (RGD) peptide (A 8052) and Arginine–Glycine–Aspartate–Serine (RGDS) (A 9041) from Sigma-Aldrich (US). Hank's Balance Salt Solution without calcium or magnesium (iHBSS) (14175-129) from Gibco-Invitrogen (US). Antibodies against glycophorin A (αGPA) (PE) (12-9987) conjugated with Phycoeryithrin (PE) and CD41 conjugated with Fluoresceine isothiocyanate (FITC) (11-0411-81) from eBioscience (US). Human fibrinogen conjugated with Alexa 488 (Fib-Alexa488) (F-13191), human fibrinogen conjugated with Alexa 647 (Fib-Alexa647) (F-35200), and calcein-AM (C-1430) from Invitrogen-Molecular Probes (US). Antibody against CD47 (αCD47) conjugated with Phycoeryithrin (556046) from BD Biosciences Pharmigen (US).

2.2. Blood collection and RBC isolation

Human blood samples were obtained by vein puncture from healthy volunteer donors at the public blood bank Instituto Português do Sangue (Lisbon, Portugal) under an institutional agreement with the Instituto de Bioquímica from Faculdade de Medicina da Universidade de Lisboa. Donors were informed and written consent was signed. This study was approved by the Ethics Committee of Faculdade de Medicina da Universidade de Lisboa. Donors were asked to give up to 9 mL of blood for research purposes additionally to their common blood donation of 450 mL to the blood bank. The blood collection was made anonymously as the data treatment resulting from the experiences made with these samples. The samples were drawn to K3EDTA anticoagulant tubes.

Briefly, a centrifugation was carried out at 200*g* for 15 min at 20 °C in a refrigerated centrifuge from Heraeus (United Kingdom), to first remove the plasma rich in platelets and the buffy coat. Then RBCs were washed three times with a solution of 0.9% NaCl at 2000 g for periods of 5 min at 20 °C. Packed RBCs were resuspended at a final concentration of $4–5\times10^9$RBC/mL with HEPES isotonic saline buffer: 133 mM NaCl, 4.5 mM KCl, 10 mM HEPES, pH 7.4.

2.3. Percoll discontinuous gradient

Human RBCs of different biological age, from healthy donors were separated as described by Corsi et al.and Venerando et al.[23,24] with some modifications. Briefly, the following solutions were prepared at room temperature (RT): HEPES-buffered stock solution (HBS stock): 2.66 M NaCl, 0.09 M KCl, 0.2 M HEPES, pH 7.4; solution A (BSA-HEPES-buffered solution, pH 7.4): 19 volumes of 5.25% (w/v) BSA in water solution added to 1 volume of HBS-stock solution; solution B (BSA-Percoll-HEPES-buffered solution, pH 7.4): 19 volumes of 5.25% (w/v) BSA in Percoll added to 1 volume of HBS-stock solution. Solutions A and B were mixed to form 5 solutions at final Percoll concentrations of 60%, 66%, 70%, 74%, and 80% (vol/vol; density 1.087–1.098 g/mL, pH 7.4). Discontinuous 5-step gradients were prepared by over layering 0.360 mL of 80%, 2.9 mL of 74%, 2.9 mL of 70%, 2.9 mL of 66%, and 1.46 mL of 60% of Percoll concentration in 15 mL tubes. A centrifugation was carried out at 2700*g* for 30 min at 20 °C to prepare de gradient. Next 1.46 mL of $4–5\times10^9$RBC/mL was layered on the top of the gradient and centrifugation was carried out at 2700*g* for 40 min. All the centrifugations were slowly decelerated over 3 min to prevent gradient disturbance. The plasma remaining above the gradient was removed, and cell fractions were collected manually over the liquid interface by aspiration from the top of the gradient using a Pasteur pipette. The fractions containing the young cells concentrated in the upper layer (over 60%), the average age cells layered over 66% and 70%, and the fractions containing the senescent cells layered over 74% were pooled carefully with a syringe and washed 3 times with HEPES-buffered isotonic saline at 2700*g* for 5 min at 20 °C. The age-dependent separation of RBCs into different fractions was confirmed by the determination of mean cell volume and mean cell haemoglobin with a cell counter (CellDyn 1600 from Abbott Laboratories). To assess that RBCs were being properly separated by this method, calcein-AM was used to confirm cell aging and viability of different fractions. Samples of total young and aged RBC were resuspended at a final concentration of 1×10^7RBC/mL with a solution of HBSS with 2%BSA pH7.4. In order to simplify from this point on total RBC will be called as RBC_T, young RBC will be called as RBC_Y and aged RBC will be called as RBC_O.

2.4. Sample preparation for flow cytometry

To study fibrinogen interaction with RBCs, these were labeled first with antibodies (according to manufacturer) and peptides (2.5 mM) for 15 min at RT in the dark. Samples were washed 2 times with iHBSS centrifugations were carried out at 1500*g* for 5 min at RT. RBC samples were resuspended at a final concentration of 1×10^7RBC/mL with HBSS pH 7.4. The different RBC populations were incubated with human fibrinogen (Alexa488- or Alexa647-labeled) (1 mg/mL) for 15 min at RT in the dark. Cells were resuspended at a final concentration of 1×10^6RBC/mL with iHBSS pH 7.4 at 4 °C, and placed in ice. Flow cytometry analyses were made in a FACSCalibur analyzer from Beckman (Germany). Settings were obtained and maintained constant throughout the experiments. Data were analyzed with the software Flow Jo.

2.5. Sample preparation for confocal microscopy

RBCs were labeled with calcein 10 μM for 45 min at 37 °C in the dark, then samples were washed two times with HBSS 2%BSA at 1500 g, 5 min at RT and resuspended 1×10^7RBCs/mL with HBSS 2%BSA. Next RBCs were labeled with antibodies and peptides for 15 min at RT in the dark, the samples were washed again two times

at 1500 g, 5 min at RT and resuspended with HBSS at final concentration 1×10^7RBCs/mL The different RBC populations were incubated with human fibrinogen–Alexa 647 (1 mg/mL) for 15 min at RT in the dark, and centrifuged at 1500*g* for 5 min at RT to remove free Fib-Alexa647. Cells were resuspended with HBSS 1%BSA at 1×10^6RBCs/mL and 50 μL of cell suspension was left to adhere to poly-L-lysine pre-coated cover-slips for 15 min at RT in the dark. The cover-slip was then mounted in slides using HBSS 1%BSA as a mount medium, and sealed with nail polish.

In a different experiment the RBCs were only labeled with fibrinogen–Alexa488 or with anti-GPA antibody and fibrinogen–Alexa488, maintaining the overall experimental procedure.

Fluorescence images were taken in a confocal microscope Zeiss LSM 510Meta from Zeiss (Germany) using an Argon/2, DPSS 561-10 and HeNe633 lasers and a 63× Plan-Apochromatic oil immersion objective. Laser and detector settings were constant within the same experiments. Images were analyzed in the computer program Image J.

2.6. Zeta-potential measurements

Zeta-potential (ζ) measurements were made in RBCs samples as described by Carvalho et al.[17], with some minor modifications. Briefly, measurements were conducted on a dynamic light scattering and zeta-potential equipment Malvern Zetasizer Nano ZS (Malvern, UK), equipped with a He–Ne laser ($\lambda = 632.8$ nm). The zeta-potential of the samples were determined, at 25 °C, from the mean of 15 measurements, with 50 runs each, with an applied potential of 30 V. The measurements were made in RBC samples pre-incubated or not with an antibody against CD47, in the absence or presence of different soluble human fibrinogen concentrations, using disposable zeta cells with platinum gold-coated electrodes (Malvern). The electrophoretic mobility obtained was used for the zeta-potential calculation through the Smoluchowski equation [25],

$$\zeta = \frac{4\pi\eta u}{\epsilon} \quad (1)$$

where u represents the electrophoretic mobility, η the viscosity of the solvent and ε its dielectric constant. The variation of the zeta-potential ($\Delta\zeta$) for each sample was calculated by subtracting from the zeta-potential value of the sample the initial value corresponding to zero fibrinogen concentration. These differences can be plotted as a function of fibrinogen concentration, and the experimental data fitted using the equation [26,27]

$$\Delta\zeta = \frac{\Delta\zeta_{max} K_L[\text{Fibrinogen}]}{1 + (K_L[\text{Fibrinogen}])} \quad (2)$$

where $\Delta\zeta_{max}$ is the maximum amplitude of variation of the zeta-potential induced by the interaction with fibrinogen, and K_L corresponds to the inverse of the value of fibrinogen concentration at $\zeta_{max}/2$.

2.7. Statistics

Flow cytometry data were statistical analyzed in Graph Prism 5. The three different populations of RBC (Geometric Mean and Median) were assessed by One or Two Way ANOVA. Significance was accepted for a P value less than 0.05.

3. Results

3.1. Fibrinogen interaction with erythrocytes

Human fibrinogen interaction with RBC membrane was studied by laser fluorescence confocal microscopy and flow cytometry. In Fig. 1 we show fluorescence confocal images that were taken from isolated erythrocytes labeled with fibrinogen–Alexa 488 (green signal) (Fig. 1A and Suppl. M1) or double labeled with an antibody against the RBC marker glycophorin A (GPA) and fibrinogen–Alexa 488 (Fig. 1B). These images show that soluble fibrinogen interact with RBCs membranes in a discrete punctuated pattern. Accordingly, we were also able to detect and quantify the interaction between soluble fibrinogen and RBCs membrane by using flow cytometry (Fig. 2A).

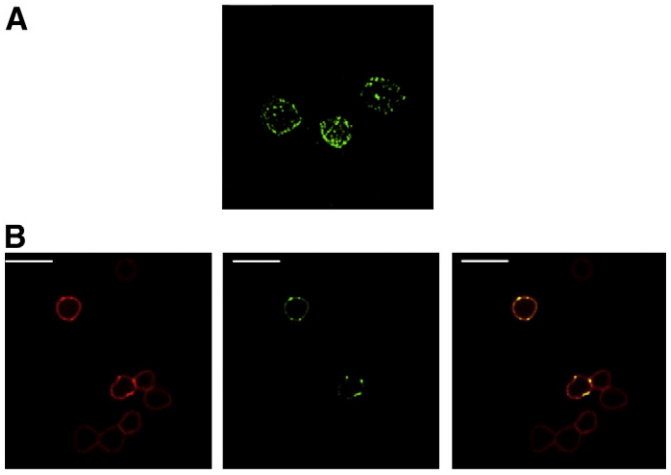


Fig. 1. Fluorescence confocal microscopy of isolated human RBC. (A and B) Human RBC were labelled with human soluble fibrinogen conjugated with Alexa488 (green signal) and/or an antibody against glycophorin A (GPA) conjugated with Phycoeryithrin (PE) (αGPA (PE)) (red signal). (A) 3D projection of fluorescent confocal microscopy images of isolated human RBC binding fibrinogen–Alexa488 (scale bar = 7 μm). Fibrinogen binding seems to occur in specific points of RBC membrane. (B) Fluorescent confocal microscopy image of isolated human RBC double labelled with fibrinogen–Alexa488 (green signal) and an anti-GPA antibody (red signal) that was used as a RBC marker (scale bar = 10 μm). We can observe that not all RBC are double labelled (yellow signal).

3.2. Fibrinogen interaction with erythrocytes is αIIbβ3 integrin and RGD/RGDS independent

Following description of the soluble fibrinogen binding with RBCs, we also decided to check if this interaction of fibrinogen with RBCs was promoted by the presence of the known fibrinogen receptor the $\alpha_{IIb}\beta_3$, For that we used a FITC-labeled antibody against CD41, a commonly used platelet marker that associates to CD61 forming the αIIbβ3 integrin receptor. Our data show that RBCs from whole blood samples did not stain for CD41 (Fig. 2). Next we addressed whether soluble fibrinogen binding to RBCs was occurring due to an integrin related receptor, by using peptides Arginine–Glycine–Aspartate (RGD) and Arginine–Glycine–Aspartate–Serine (RGDS) in a competition study, a commonly used method to study fibrinogen interaction with integrin-like receptors [15]. Our data indicate that the presence of peptides RGD or RGDS did not significantly affect the interaction of RBCs with soluble fibrinogen (Fig. 3).

3.3. Age dependency of fibrinogen interaction with erythrocytes

In the microscopy experiments, we noticed that not all RBCs were able to bind fluorescently labeled fibrinogen with similar intensity (Fig. 1B). This fact led us to hypothesize that interaction with soluble fibrinogen might be dependent on the RBC age. In this respect, we investigated fibrinogen binding to RBC populations with different ages.

The positive labeling with anti-GPA antibody (αGPA) was used to facilitate the identification of three different populations of RBCs in flow cytometry experiments: (i) total RBCs designed as RBC_T (Fig. 4A), (ii) young RBCs as RBC_Y (Fig. 4B), and (iii) old RBCs

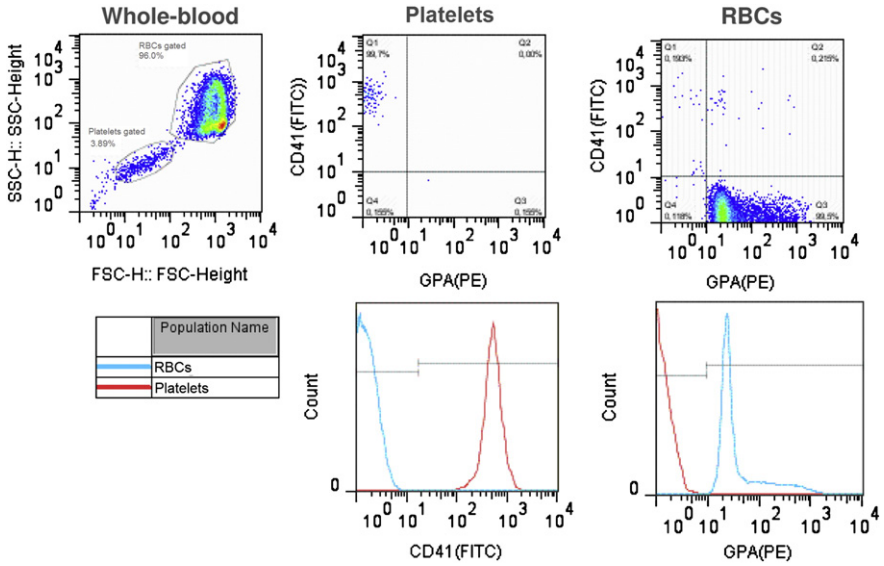


Fig. 2. Flow cytometry of whole human blood. Subpopulations of Platelets and RBC were labelled with an anti-CD41 (FITC) and anti-GPA (PE) respectively. For each subpopulation we performed SSC/FSC as well as CD41 (FITC) /αGPA (PE) plots. As expected our data show clearly that CD41 (FITC) positive labelling was presented in platelet population (99.7%) and GPA (PE) in RBC population (99.5%). A very small percentage (0.2%) shows a positive labelling both for CD41 and GPA in RBC gated population, which could be due to some platelets aggregated to RBCs.

identified as RBC_O (Fig. 4C). As published by others [28,29] antibodies against GPA promote RBC agglutination (notice the dispersion of different RBCs populations in SSC/FSC plots as well as the formation of smallest peaks in the fluorescence histogram for αGPA (Fig. 5A), thus we limited its utilization to initial setup experiments. Nevertheless, we found that RBC agglutination was not detrimental towards binding of soluble fibrinogen with erythrocytes.

Calcein-AM was used to confirm the age and viability of the different RBC populations separated by the Percoll discontinuous gradient. This probe is a highly lipophilic vital dye that passively enters viable

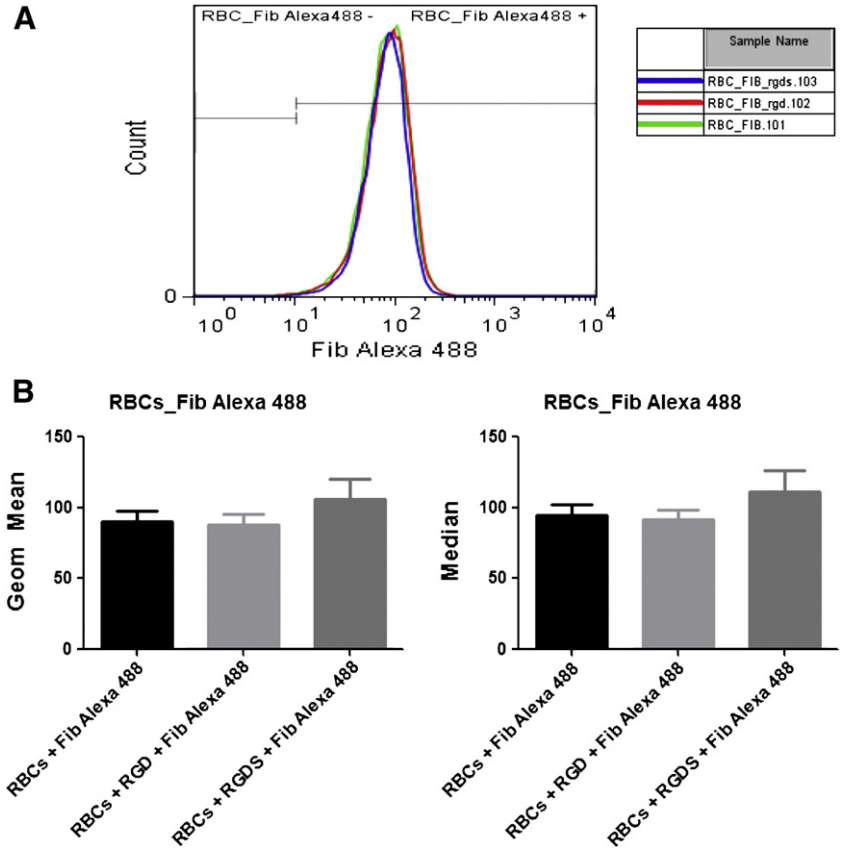


Fig. 3. Flow cytometry histogram and analysis from isolated human RBCs labeled with Fib-Alexa488 in presence of RGD and RGDS. (A) Flow cytometry histograms for RBC_Fib Alexa488-RGD, RBC_Fib Alexa488-RGDS and RBC_Fib Alexa488. The interaction with human soluble fibrinogen was not significantly affected ($P > 0.05$) neither due to the presence of RGD or RGDS. (B) Analysis of geometric mean and median values for the different samples. The interaction of soluble Fib-Alexa488 with RBC was not affected in the presence of RGD and RGDS.

cells, and is converted by intracellular esterases to calcein that produces an intense green signal, cells with intact plasma membrane are able to retain this probe [30]. As shown in supplemental figure S2, calcein fluorescence in young RBCs was higher than that in old RBCs due to the increased esterase activity, which means that Percoll discontinuous gradient is a good method to separated RBCs according to age.

In flow cytometry (Fig. 5A), we observed that fibrinogen interacts more with young RBCs than with older RBCs. The geometric mean and median of fluorescence (Fig. 5B) were statistically analyzed and confirm that fibrinogen interaction with young RBCs is significantly higher ($P<0.01$) than with aged RBCs.

Next we made use of fluorescence confocal microscopy to test our working hypothesis. In Fig. 6A we present images taken from the three different populations of RBCs labeled with calcein (blue signal) and fibrinogen–Alexa647 (red signal). We can observe a higher interaction of fibrinogen–Alexa 647 with RBC_Y subpopulation than with RBC_T, and importantly notice the absence of fibrinogen–Alexa 647 labeling in the RBC_O subpopulation (Fig. 6A).

3.4. CD47 mediates differential binding of soluble fibrinogen to younger and old erythrocytes

The differential binding of soluble fibrinogen to subpopulations of erythrocytes led us to look closer at membrane proteins involved in erythrocyte survival and removal from the circulation. From several potential targets CD47 was an obvious one, since it is directly involved in these processes [31,32].

The role of CD47 in the interaction of the erythrocyte membrane with soluble fibrinogen was studied by flow cytometry of different RBCs populations (Fig. 7A). For this experiment erythrocytes were previously incubated with an antibody against CD47 (αCD47), before fibrinogen–Alexa488 addition. As published by others, this antibody also promotes RBC agglutination [33,34]. We confirmed this with the same strategy that we previously used for anti-GPA, by observing the dispersion of the different RBC subpopulations in SSC/FSC plots (data not shown) as well as the formation of smallest peaks in histogram for αCD47 (Fig. 7A). RBCs with different biological ages have shown to have similar CD47 levels (Fig. 7A).

Strikingly, the interaction of soluble fibrinogen with the RBC membrane is significantly decreased by blocking CD47 in its surface. The flow cytometry experiments reveal that the interaction of soluble fibrinogen–Alexa 647 with RBC membrane is diminished in all samples and much more evident for the case of the younger RBCs (Fig. 7A). In the presence of the anti-CD47 antibody the fibrinogen–Alexa 647 binding becomes similar for all the three samples. Apparently young RBCs lose their ability to bind more soluble fibrinogen than other RBCs subpopulations when we block CD47. Flow cytometry control experiments with isotype antibodies for αCD47 and αGPA were made (Suppl. S3).

We also obtained fluorescence confocal microscopy images of RBCs pre-incubated with αCD47, and then with fibrinogen–Alexa647 (Fig. 6B). In the presence of anti-CD47 antibody we observed no evidence of binding of soluble fibrinogen–Alexa647 to any of the RBCs subpopulations indicating that this anti-CD47 antibody is acting as a blocker for fibrinogen–Alexa647 binding to the erythrocyte membrane.

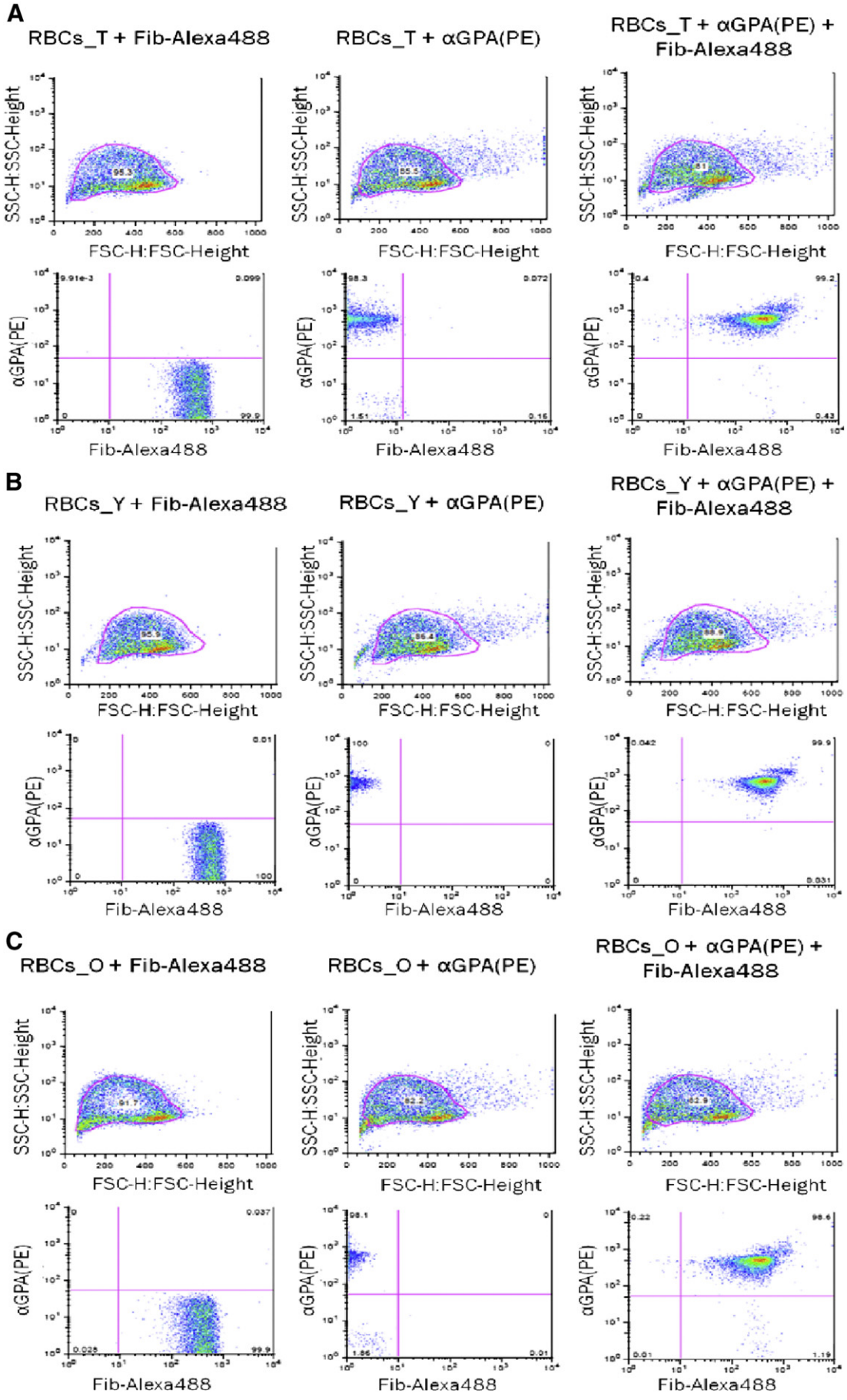
To confirm our previous data we decided to make use of a different type of approach, so we performed zeta-potential measurements in RBCs pre-incubated with the anti-CD47 antibody in absence or presence of 1 and 3 mg/mL of human soluble fibrinogen. The zeta-potential measurements show us once more that the presence of the anti-CD47 antibody diminishes the ability of young blood cells to bind soluble fibrinogen, reaching a similar level observed for the old RBCs. The extension of the fibrinogen–young RBCs interaction obtained, $\Delta\zeta_{max} = (7.4 \pm 1.4)$ mV, is significantly higher ($P<0.001$) than the values obtained for young RBCs in the presence of anti-CD47 $\Delta\zeta_{max} = (3.5 \pm 0.8)$ mV, old RBCs $\Delta\zeta_{max} = (2.5 \pm 1.2)$ mV and old RBCs in the presence of anti-CD47 $\Delta\zeta_{max} = (2.4 \pm 1)$ mV (Fig. 8).

4. Discussion

It has been previously proposed that RBC hyperaggregation may occur as a result of soluble fibrinogen binding that would take place due to non-specific and specific mechanisms [13–17]. Some studies actually point out that fibrinogen alpha chain might be the possible binding site of this molecule to RBC membrane [13,14]. Additionally, recent studies using biophysical approaches have tried to better understand the possible fibrinogen–erythrocyte interaction [16,17]. Although we agree that soluble fibrinogen may interacts non-specifically and specifically with erythrocyte membranes, from our point of view these studies needed to be complemented and clarified by a different approach, so we tried to elucidate this issue by developing a simple and fresh strategy mainly based on *in situ* experiments and molecular cell biology methodologies. Our main goals were to prove that human RBCs are able to specifically bind soluble fibrinogen, and additionally identify membrane molecular targets that could be involved in this process. So, we have used a set of experiments that include flow cytometry and fluorescence confocal microscopy methodologies, in order to quantify and image (for the first time) the soluble fibrinogen–erythrocyte interaction, and to support our findings we also have used zeta-potential measurements, as a biophysical approach that was recently established and published by Carvalho et al. [17] to study this theme.

Platelets and platelet-microparticules are enriched in αIIbβ3, a well-known and studied receptor of soluble fibrinogen. Furthermore, the presence of platelets or platelet microparticules (MP) adhered to RBCs membrane is possible in circulation. So, to address whether fibrinogen–erythrocyte interaction is not occurring as a result of αIIbβ3 due to its presence in RBCs-platelets or platelets MP aggregates, we used CD41 immunostaining to perform flow cytometry measurements (CD41 is a commonly used platelet marker and indicator of glycoprotein αIIbβ3 presence). Our data showed no evidence for the presence of this platelet specific- integrin in the RBCs population. So these results, together with the fluorescent confocal microscopy images (Fig. 1A, Suppl. S4 and M1), made us to conclude that the fibrinogen–erythrocyte interaction is not occurring due to $\alpha_{IIb}\beta_3$ presence from aggregated platelets or platelet-microparticules to the RBCs membrane.

Lominadze et al. [15] using murine RBCs and ghosts, reported that fibrinogen binding to RBCs membrane and aggregation due to its presence, was blocked by Arginine–Glycine–Aspartate–Serine (RGDS) peptide, which indicated that the specific binding of fibrinogen to RBCs could occur due to an integrin-like receptor. Additionally, Carvalho et al. [16] compares the fibrinogen–platelet interaction with the fibrinogen–erythrocyte interaction in her AFM study. This study starts to report that the receptor should be $\alpha_{IIb}\beta_3$-like because, according to their data, this interaction was not so strongly influenced by calcium and eptifibatide (a specific inhibitor of $\alpha_{IIb}\beta_3$). At the end they claimed that it as to be a related β_3 integrin-like receptor, supporting their findings in just one case from a patient with Glanzmann thrombasthenia (a rare hereditary bleeding disorder caused by a $\alpha_{IIb}\beta_3$ deficiency) that had a mutation in β_3 subunit. Notice that the atomic force microscopy approach employed in the mentioned study uses tips coated with adsorbed fibrinogen; since adsorbed fibrinogen changes its conformation and exposes previously hidden epitopes, interaction reports from adsorbed fibrinogen may not be a faithful representative of the interactions established by the soluble molecule. Both studies report [15,16] that specific soluble fibrinogen–erythrocyte interaction occurs due to an integrin-like receptor, so it should be Arginine–Glycine–Aspartate (RGD) and Arginine–Glycine–Aspartate–Serine (RGDS) dependent. These two sequences are located at fibrinogen alpha chain and both are responsible for the


A
RBCs_T + Fib-Alexa488
RBCs_T + αGPA(PE)
RBCs_T + αGPA(PE) + Fib-Alexa488
SSC-H:SSC-Height
FSC-H:FSC-Height
αGPA(PE)
Fib-Alexa488
B
RBCs_Y + Fib-Alexa488
RBCs_Y + αGPA(PE)
RBCs_Y + αGPA(PE) + Fib-Alexa488
C
RBCs_O + Fib-Alexa488
RBCs_O + αGPA(PE)
RBCs_O + αGPA(PE) + Fib-Alexa488

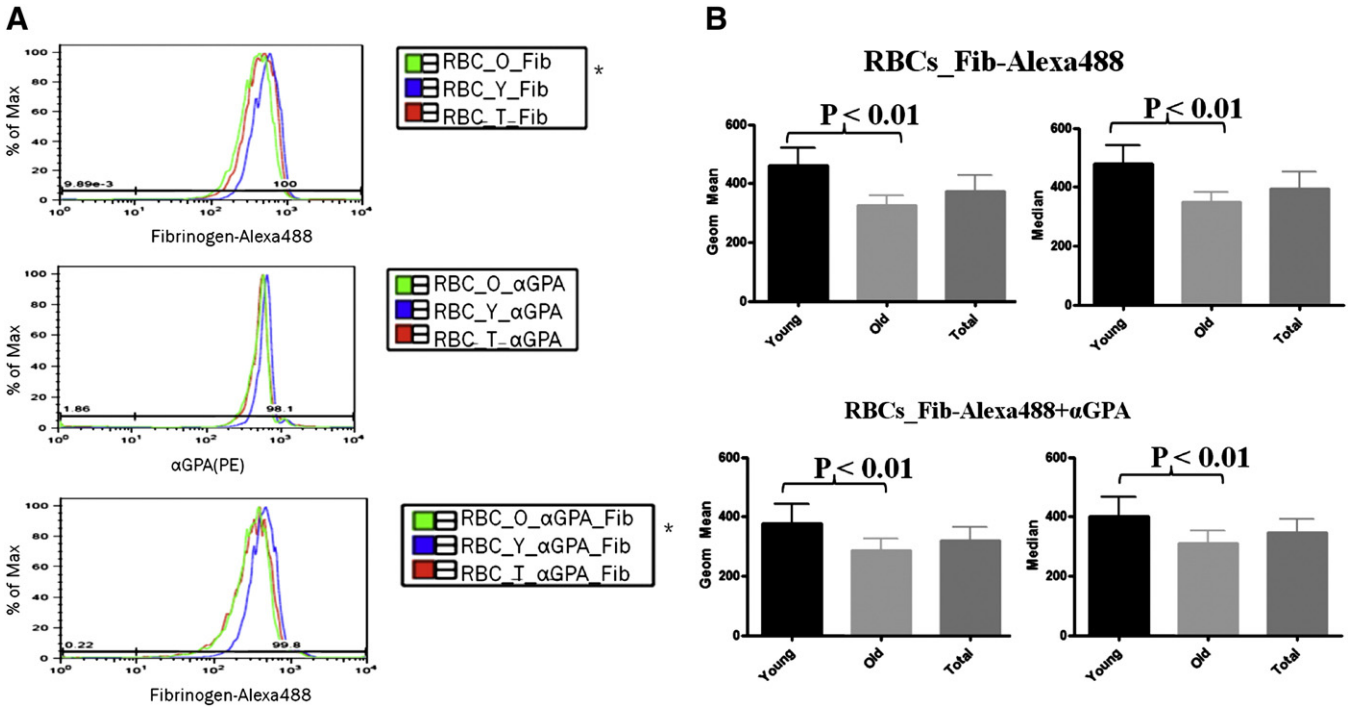


Fig. 5. Analysis of flow cytometry from RBC labelled with Fib-Alexa488 and/or αGPA (PE). (A) Flow cytometry histograms for Total RBC (RBC_T), Young RBC (RBC_Y) and Old RBC (RBC_O), labelled with Fib-Alexa488 and/or αGPA (PE). The interaction with human soluble fibrinogen was significantly higher ($P<0.01$) in RBC_Y than in the other two RBC subpopulations (*$P<0.01$). (B) Analysis of geometric mean and median values for the different RBC subpopulations labelled with Fib-Alexa488 and/or αGPA (PE). Significant different values for geometric mean and median ($P<0.01$) are found between the interaction of soluble Fib-Alexa488 with young RBC and with old RBC. Notice that there were no significant differences between RBC + Fib-Alexa488 and RBC + Fib-Alexa488 + αGPA, which shows that αGPA(PE) does not interfere with fibrinogen interaction with RBC membrane.

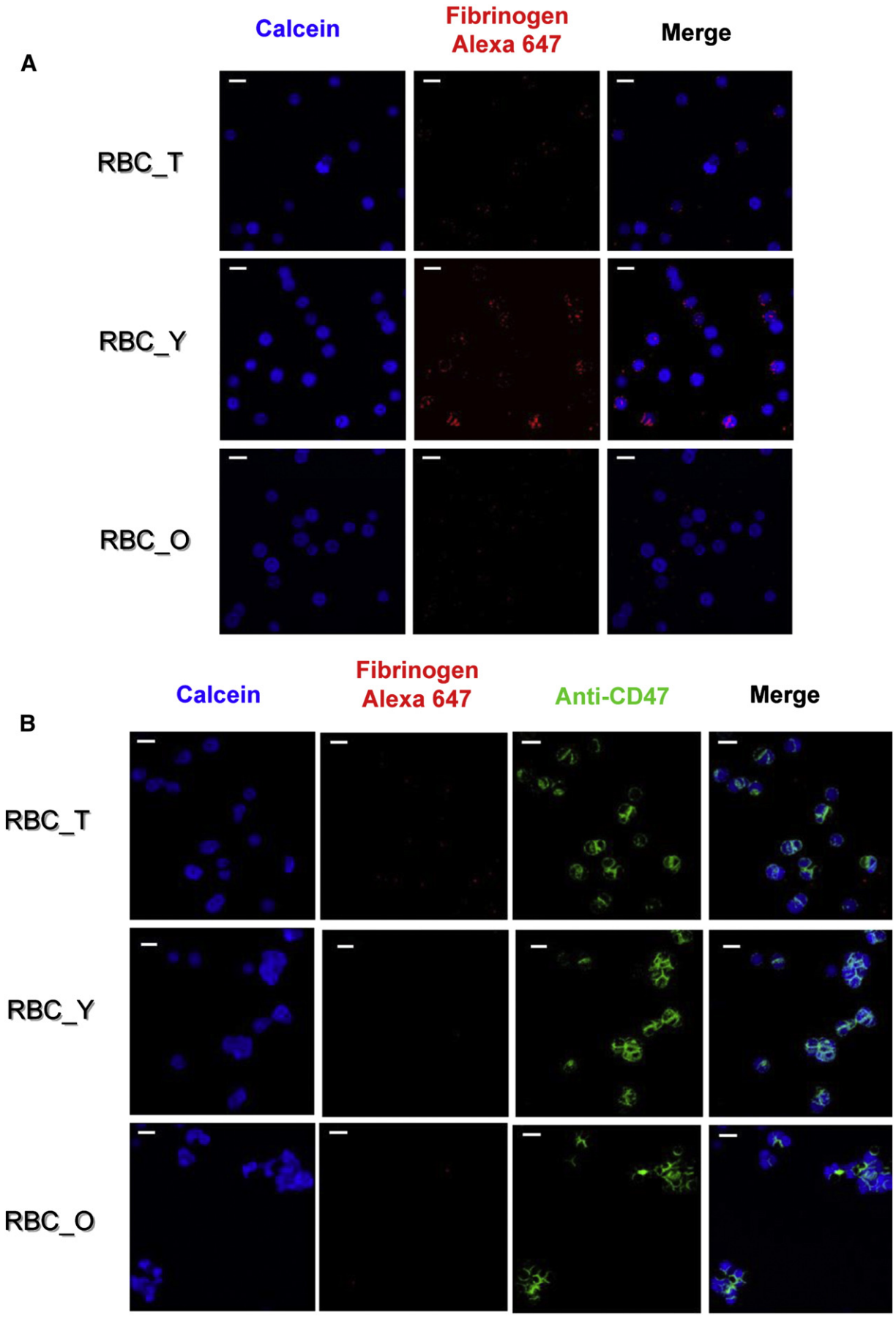
low intensity interaction with integrin-like receptors. Our results indicate that the presence of peptides RGD/RGDS do not inhibit the soluble fibrinogen binding to human RBCs neither the erythrocyte aggregation (data not shown), contrary to what it was reported by Lominadze et al. using murine RBCs/ghosts [15]. So, according to this there are no data to support the idea that soluble fibrinogen specific binds to RBCs as a result of an integrin-like receptor.

As we mentioned before the interaction between soluble fibrinogen and RBC membrane was already studied and verified by others, although we present for the first time fluorescent confocal microscopy images of it. From the beginning we noticed that this interaction seems to occur in specific points of the membrane, and that not all RBCs appear to be able to equally bind fibrinogen. This fact had led us to hypothesize that the variations on fibrinogen–erythrocyte interactions could be age dependent. Knowing that the isoelectric point of fibrinogen is around 5.1–5.5, being negatively charged under physiological conditions (pH 7.4), and also that aged RBCs have a less negatively charged membrane [35,36] consequently with an higher aggregation index [7,11,15,17,37–40], we expected that fibrinogen would interact more with old RBCs in comparison with the young ones. But surprisingly the opposite occurs. All our approaches have confirmed that soluble fibrinogen interacts more extensively with younger RBCs than with the old ones, suggesting that erythrocyte binding affinity for fibrinogen is surpass but not dependent of RBC membrane external charge (Figs. 5, 6A and 8). So our data confirm the recently published idea that fibrinogen–erythrocyte interaction is age-dependent [17].

All the data collected until this point prompted us to further evaluate the possibility for the existence of a specific molecular target at the RBCs membrane for soluble fibrinogen. Actually, RBCs have a wide range of surface molecules that could be responsible for this interaction such as CD47, B-CAM, VLA-4 and glycosphingolipids (gangliosides) [18,19,41–43]. From all these targets, CD47 was a very strong candidate due to several characteristics. For example, in RBCs this integrin-associated protein is involved in diverse biological functions, it is part of the Rh macrocomplex which is connected to the cytoskeleton via protein 4.2, and interacts also with the major erythrocytic macrocomplex Band 3 that among other functions is involved in RBC aggregation [44–47]. CD47 is also a molecular target for several proteins such as thrombospondin, laminin, and fibronectin [48,49]. Besides all this, CD47 is implicated in erythrocyte clearance by phagocytes. On healthy cells CD47 binds to the tyrosine kinase signal regulatory protein α (SIRPα) [45,46,50,51], this engagement is a key “don't eat me” signal from healthy cells to phagocytes [50]. RBCs are cleared from the bloodstream mainly in the spleen by splenic macrophages due to a specific mechanism that among several other factors also involves CD47. The blocking of CD47-SIRPα signal or the loss of CD47 expression is one of the mechanisms that cause the removal of non-aged erythrocytes from the bloodstream [51].

In order to identify potential membrane ligands or mediators for soluble fibrinogen binding, we addressed the involvement of the above mentioned targets by using several different approaches that could block the interaction of fibrinogen to RBCs membrane. Strikingly, only an antibody against human CD47 was able to significant decrease the

Fig. 4. Flow cytometry of isolated human RBC with different biological ages. Subpopulations of RBC were labeled with an anti-GPA antibody and fibrinogen Alexa488 (Fib-Alexa488). For each RBC subpopulation we performed SSC/FSC as well as αGPA (PE)/Fib-Alexa488 plots; Total RBC (RBC_T) (A), Young RBC (RBC_Y) (B) and Old RBC (RBC_O) (C). In each set of data the first and second column are controls for single stainings of each probe: Fib-Alexa488 or αGPA (PE). The third column (see αGPA (PE)/Fib-Alexa488 plots) shows double positive labelled RBC with Fib-Alexa488 and αGPA (PE). All RBC subpopulations (RBC_T, RBC_Y and RBC_O) are double positive labelled. Additionally, in the SSC/FSC plots we observe that the anti-GPA antibody (second and third column) increases the events dispersion due to an agglutination phenomenon that is responsible for the formation of RBC duplets and triplets.


A
Calcein
Fibrinogen Alexa 647
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RBC_T
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B
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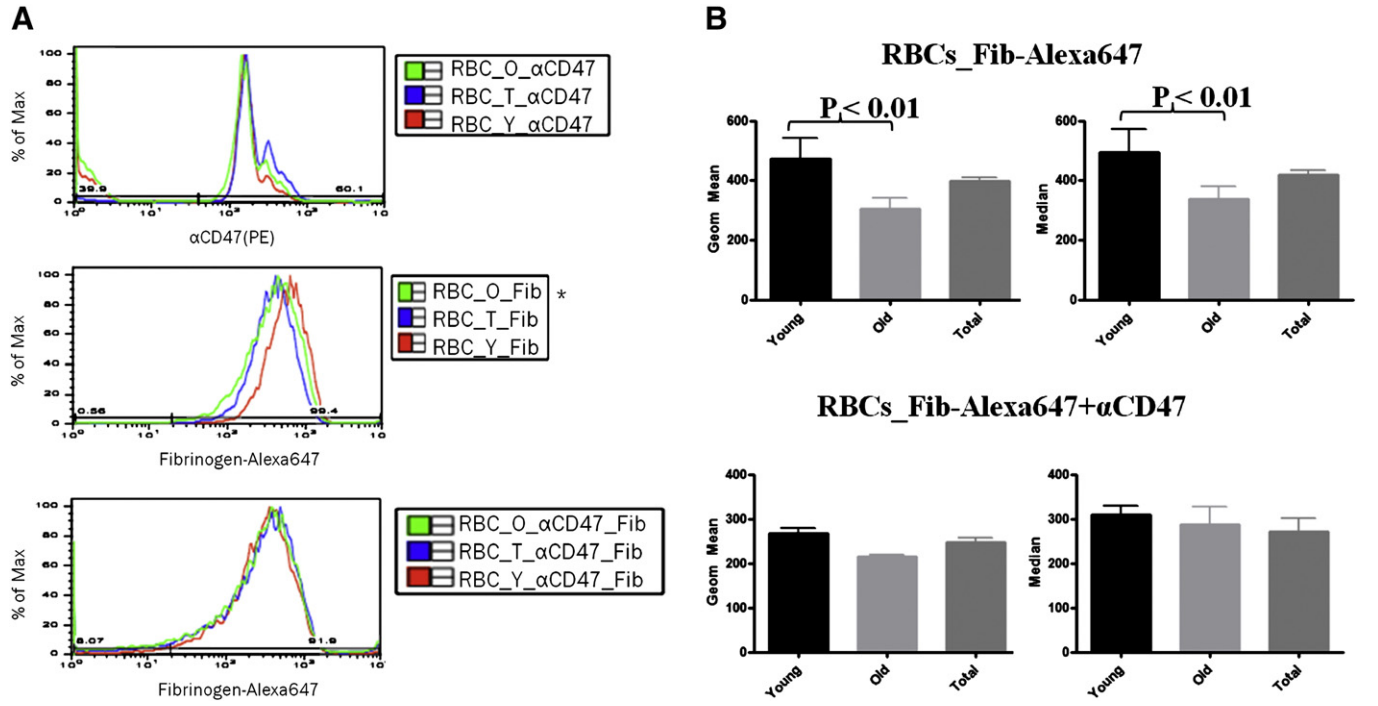

Fig. 7. Analysis of flow cytometry from RBC labelled with Fib-Alexa647 and/or antibody against CD47 conjugated with Phycoeryithrin (PE) (αCD47). RBC with different biological ages (RBC-T, RBC-Y and RBC-O) were labelled with human soluble fibrinogen conjugated with Alexa 647 (Fib-Alexa647) and αCD47 conjugated with PE (αCD47). (A) Flow cytometry histograms for each RBC sample. No significant differences in CD47 levels were found between the three RBC subpopulations. Notice that in the presence of anti-CD47 antibody the soluble fibrinogen interacts similarly with the three RBC populations, which suggests that this antibody is blocking the interaction of Fib-Alexa647 with the membrane of younger RBC. (B) Analysis of geometric mean and median values. Significant different values for geometric mean and median (*$P<0.01$) are found between the interaction of soluble fibrinogen Alexa647 with young RBC and with old RBC. The RBC Fib-Alexa647 and RBC Fib-Alexa647 + αCD47 were significantly different ($P<0.05$) for both parameters analyzed.

soluble fibrinogen binding to RBCs. The fluorescence confocal microscopy images also show us that the antibody against CD47 inhibits the interaction between subpopulation of young and total RBCs and soluble fibrinogen, to a similar level obtained for the old RBCs subpopulation. To better evaluate this we performed zeta-potential measurements by using a similar approach recently published by Carvalho et al. [17]. Zeta-potential measurements can be used to determine the extension of the interaction between two particles, by calculating the values of $\Delta\zeta_{max}$. Young RBCs have a higher extensive interaction with soluble fibrinogen than the old ones. So we have determined the extension of the interaction between pre-incubated RBCs with antibody against CD47and human soluble fibrinogen. The values obtained clearly show us that the presence of the anti-CD47 antibody diminish the ability of young RBCs to interact with fibrinogen, $\Delta\zeta_{max} = (7.4 \pm 1.4)$ mV, this value is significantly ($P<0.001$) higher than the values obtained for young RBCs in the presence of anti-CD47, $\Delta\zeta_{max} = (3.5 \pm 0.8)$ mV, old RBCs, $\Delta\zeta_{max} = (2.5 \pm 1.2)$ mV, and old RBCs in the presence of anti-CD47, $\Delta\zeta_{max} = (2.4 \pm 1)$ mV. Additionally, as we did not find significant differences in CD47 levels between the three subpopulations of RBCs and as CD47 might have different conformations [48–51] it is tempting to speculate that soluble fibrinogen might interact more with younger erythrocytes due to conformation changes of the protein that may occur during the cell ageing process. Overall this set of experiments seems to point out CD47 as a putative mediator for soluble fibrinogen interaction with human RBCs. Further experiments will be needed to address with more accuracy if CD47 is just a putative mediator or if it is playing a more important role in this specific interaction.

In summary, we report here that soluble fibrinogen specifically interacts with the erythrocyte membrane in what it seems a non-integrin like receptor, and we also confirm that this interaction is age-dependent. We also point out CD47 as a putative mediator for the soluble fibrinogen binding to human erythrocyte membrane. Knowing that erythrocyte hyperaggregation promoted by high levels of fibrinogen may lead to severe and deathly scenarios in patients that suffers from several diseases, we believe that this work has taken the initial steps in order to use erythrocytes and CD47 as novel targets in the development of new therapeutical strategies as an option to the already existing ones that mainly target platelets and its receptors.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2011.10.028.

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Fig. 6. Fluorescence confocal microscopy of isolated human RBC. (A) Fluorescent confocal microscopy of different RBC subpopulations double labelled with calcein (blue signal) and fibrinogen–Alexa647 (red signal). RBC subpopulations are referred as: Total (RBC-T), Young (RBC-Y) and Old (RBC-O). In this image we can observe that just two RBC subpopulations present a positive labeling for fibrinogen–Alexa 647, which are the RBC-T and RBC-Y. We were not able to observe a positive labelling for soluble fibrinogen–Alexa 647 from the RBC-O subpopulation (scale bar = 10 μm). (B) Fluorescent confocal microscopy of RBC labelled with calcein (blue signal), fibrinogen–Alexa647 (green signal) and antibody against CD47 conjugated with Phycoeryithrin (PE) (αCD47). We can observe that fibrinogen–Alexa647 does not interact with the membrane of either RBC subpopulations, the presence of the anti-CD47 antibody seems to block the interaction of soluble fibrinogen with the RBC membrane (scale bar = 10 μm).

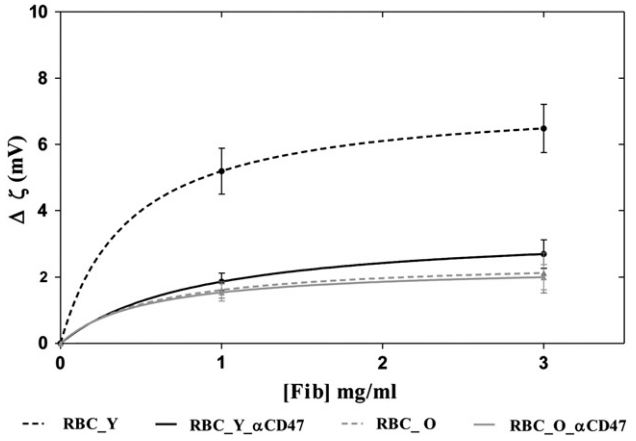

Fig. 8. Variations of zeta-potential. The extension of the interaction between young RBCs and soluble fibrinogen was higher ($\Delta\zeta_{max} = (7.4 \pm 1.4)$ mV) than in any of the other samples (RBC_Y_αCD47, $\Delta\zeta_{max} = (3.5 \pm 0.8)$ mV, RBC_O, $\Delta\zeta_{max} = (2.5 \pm 1.2)$ mV, and RBC_O_αCD47, $\Delta\zeta_{max} = (2.4 \pm 1)$). Statistically significant differences ($P < 0.001$) were found between the samples.

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