Phosphatidylserine, an anionic phospholipid normally present exclusively on the inner leaflet of the membrane bilayer is exteriorized to the outer leaflet in a small percentage of sickle red blood cells and is thought to be a tag for macrophage recognition. Lactadherin, secreted by macrophages, promotes the engulfment and clearance of phosphatidylserine-expressing apoptotic cells. We isolated lactadherin from bovine milk and labeled it with fluorescein isothiocyanate (FITC). Lactadherin incorporated 1.8 mole of fluorescein/mole as determined by the \( \varepsilon \) of 78,000 M\(^{-1}\) cm\(^{-1}\). Red blood cell suspensions were prepared from fresh human blood collected from laboratory volunteers or patients with sickle cell anemia following informed consent. All the samples were from adult patients (aged 23-51 years) with homozygous SS disease. These patients were seen in the out-patients clinic. None of the patients was clinically in a pain crisis at the time of the study.

Lactadherin binding to red cells was analyzed on a flow cytometer (Coulter EPICS XL, Beckman-Coulter Inc., San Jose, CA, USA) following incubation with FITC-lactadherin (100 nM). Acquisition and data analysis were performed using EXPO32 software. Ten thousand events per sample were acquired to ensure adequate mean fluorescence levels. The light scatter and fluorescence channels were set at a logarithmic gain. Less than 0.5% of the normal red blood cells showed any binding. In contrast, the sickle red blood cells from nine different individuals bound two to five-fold more FITC-lactadherin (Figure 1, \( p<0.0002 \) when analyzed by Wilcoxon’s matched pairs signed ranks test). FITC-lactadherin binding was inhibited by a 50-fold molar excess of unlabeled lactadherin, annexin V or phosphatidylserine vesicles. These data support the concept that a proportion of circulating red blood cells in sickle cell anemia patients express phosphatidylserine on their outer surface.

We next studied the effect of lactadherin on the phagocytosis of red blood cells by peripheral blood monocyte-derived macrophages. When normal red blood cells were incubated with macrophages under basal condition, a few cells bound to the surface of the macrophages. However, in the presence of lactadherin a significantly higher proportion of sickle red blood cells than normal red blood cells were bound to the macrophages. These red cells were rapidly phagocytosed, as observed under a phase contrast microscope. To quantify phagocytosis, red blood cells were incubated with monocyte-derived macrophages in a ratio of 1000:1 for 90 minutes at 37°C and the surface bound red blood cells were lysed with hypotonic saline and washed. The internalized cell-associated hemoglobin was determined by its pseudoperoxidase activity as described elsewhere. In the presence of lactadherin a significantly higher proportion of sickle red blood cells than normal red cells were phagocytosed (Figure 2A). We also observed that antibodies to integrin \( \alpha_V\beta_3 \) inhibited the phagocytosis of phosphatidylserine-expressing red blood cells while a monoclonal antibody to Fc\( \gamma \) receptor II (IV3) had no significant effect under similar conditions (Figure 2B). These results indicate that the lactadherin-induced phagocytosis is mediated by integrin \( \alpha_V\beta_3 \) on macrophages and that the Fc\( \gamma \) receptor does not play a major role.

The shortened red blood cell survival in sickle cell anemia is due to extravascular destruction mediated by the macrophages. Several mechanisms, including the exposure of phosphatidylserine, have been proposed as tags for macrophage recognition. Lactadherin is present in small amounts in blood and macrophages secrete lactadherin when stimulated. This may especially relevant in the spleen and liver where large numbers of macrophages encounter red blood cells. The spleen, which has plentiful macrophages, enlarges early in the life of sickle cell
anemia patients due to an accumulation of red cells. Slow and sluggish blood flow in the splenic pulp predisposes to a lower oxygen tension, promoting the exposure of phosphatidylserine and engulfment of the cells by the splenic macrophages. Eventually, the spleen undergoes atrophy due to ongoing vascular occlusions. However, early in life, in some children with sickle cell disease the enlarged spleen may become engorged with massive volumes of red blood cells leading to peripheral circulatory failure (acute sequestration crisis). This life-threatening complication is seen in association with infection; however, the explanation for this dreaded complication is unknown. Increased lactadherin secreted by stimulated macrophages in the spleen during infections may play a role in acute sequestration crisis.

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Figure 2. Panel A. Effect of lactadherin on the phagocytosis of red blood cells by macrophages. Washed red blood cells from a healthy volunteer and five patients with sickle cell anemia were incubated with monocyte-derived macrophages with and without lactadherin (100 nM) and the engulfed red cells were quantified by measuring the cell associated pseudoperoxidase activity of hemoglobin. The mean ± s.e.m of triplicate measurements are shown. The difference between phagocytosis in the presence or absence of lactadherin was significant (p<0.01) in all sickle patients. Panel B. Effect of antibodies on lactadherin-induced phagocytosis of red cells. Ten µg/mL of abciximab (anti-Integrin αVβ3), control antibody (rituximab, anti-CD20), or IIV3 (anti-Fcγ receptor II) were incubated with the macrophages and the effect of lactadherin-induced phagocytosis was determined as above. The phagocytosis in the absence of any antibodies was considered 100%.