



## Phosphatidylserine Receptor Is Required for Clearance of Apoptotic Cells

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# Phosphatidylserine Receptor Is Required for Clearance of Apoptotic Cells

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Cells undergoing apoptosis during development are removed by phagocytes, but the underlying mechanisms of this process are not fully understood. Phagocytes lacking the phosphatidylserine receptor (PSR) were defective in removing apoptotic cells. Consequently, in PSR-deficient mice, dead cells accumulated in the lung and brain, causing abnormal development and leading to neonatal lethality. A fraction of PSR knockout mice manifested a hyperplastic brain phenotype resembling that of mice deficient in the cell death-associated genes encoding Apaf-1, caspase-3, and caspase-9, which suggests that phagocytes may also be involved in promoting apoptosis. These data demonstrate a critical role for PSR in early stages of mammalian organogenesis and suggest that this receptor may be involved in respiratory distress syndromes and congenital brain malformations.

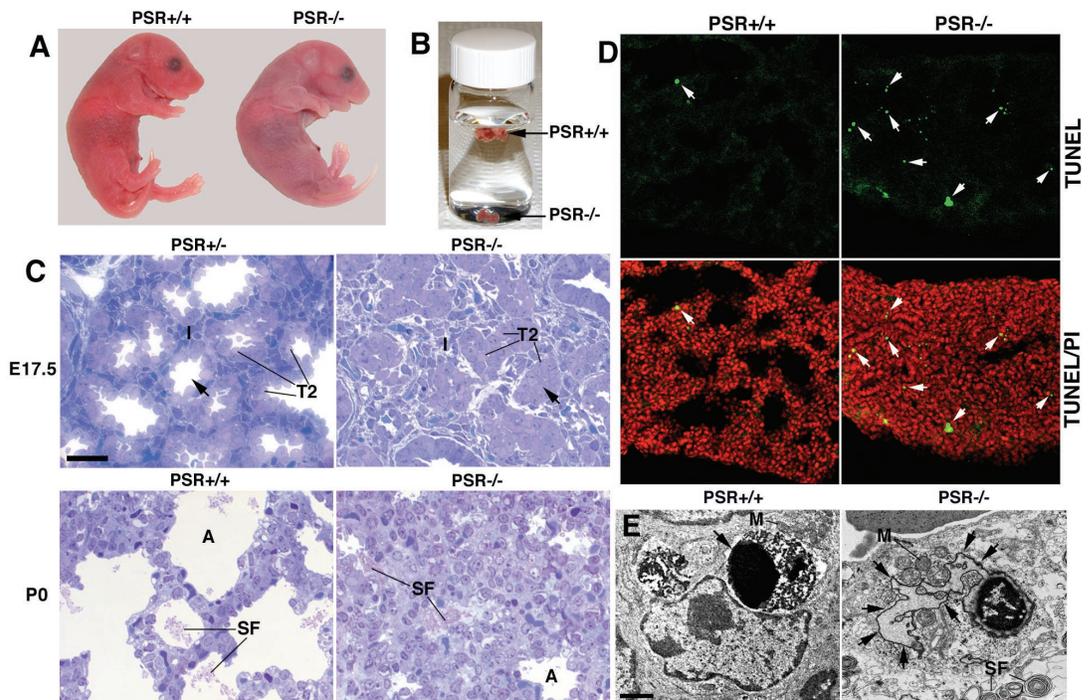
ty lipoprotein receptor-1 (LOX-1),  $\beta_2$ -glycoprotein I ( $\beta_2$ GPI) receptor,  $\alpha_v\beta_3$  vitronectin receptor, Mer receptor tyrosine kinase, and PSR (4–9). In vitro, PSR is essential for the engulfment of apoptotic cells by both professional and amateur phagocytes, including macrophages, fibroblasts, epithelial cells, and endothelial cells (9, 10). We examined PSR expression in mouse embryos. Northern blot analysis indicated that PSR is expressed as early as embryonic day 7 (E7) (fig. S1A). PSR transcripts were present in multiple tissues including brain, eye, spinal cord, thymus, lung, liver, kidney, and intestine (fig. S1B).

To delete PSR in the mouse, we replaced the first two exons of the PSR gene with the neomycin resistance gene in mouse embry-

Exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane of apoptotic cells is considered a primary signal

recognizable by phagocytes (1–3). Several receptors are implicated in the recognition of PS, including lectin-like oxidized low-densi-

**Fig. 1.** Abnormal lung development and failed clearance of apoptotic cells in PSR-deficient mice. (A) Phenotype of PSR<sup>+/+</sup> and PSR<sup>-/-</sup> mice at P0. PSR<sup>-/-</sup> mice showed cyanotic skin color. (B) Lung from a P0 PSR<sup>-/-</sup> mouse sinks in phosphate-buffered saline; lung from a PSR<sup>+/+</sup> mouse floats. (C) Reduced luminal air spaces in PSR<sup>-/-</sup> lung at E17.5 (upper panels, arrows) and P0 (lower panels, A). At E17.5, expanded interstitial areas (I) are between type II epithelial cells (T2); at P0, lung surfactant (SF) is indicated in PSR<sup>+/+</sup> and PSR<sup>-/-</sup> lungs. (D) Increased TUNEL-positive cells (arrows) in E17.5 PSR<sup>-/-</sup> lungs. Counterstaining with propidium iodide (PI) is also shown (lower panels). (E) EM analysis of an engulfed apoptotic cell (arrow) in PSR<sup>+/+</sup> lung (left). A nonphagocytosed, necrotic-like cell with a semicondensed nucleus and dilated nuclear membranes (arrows) and mitochondria (M) in PSR<sup>-/-</sup> lung (right). Scale bars, 25  $\mu$ m (C), 2.5  $\mu$ m (E).



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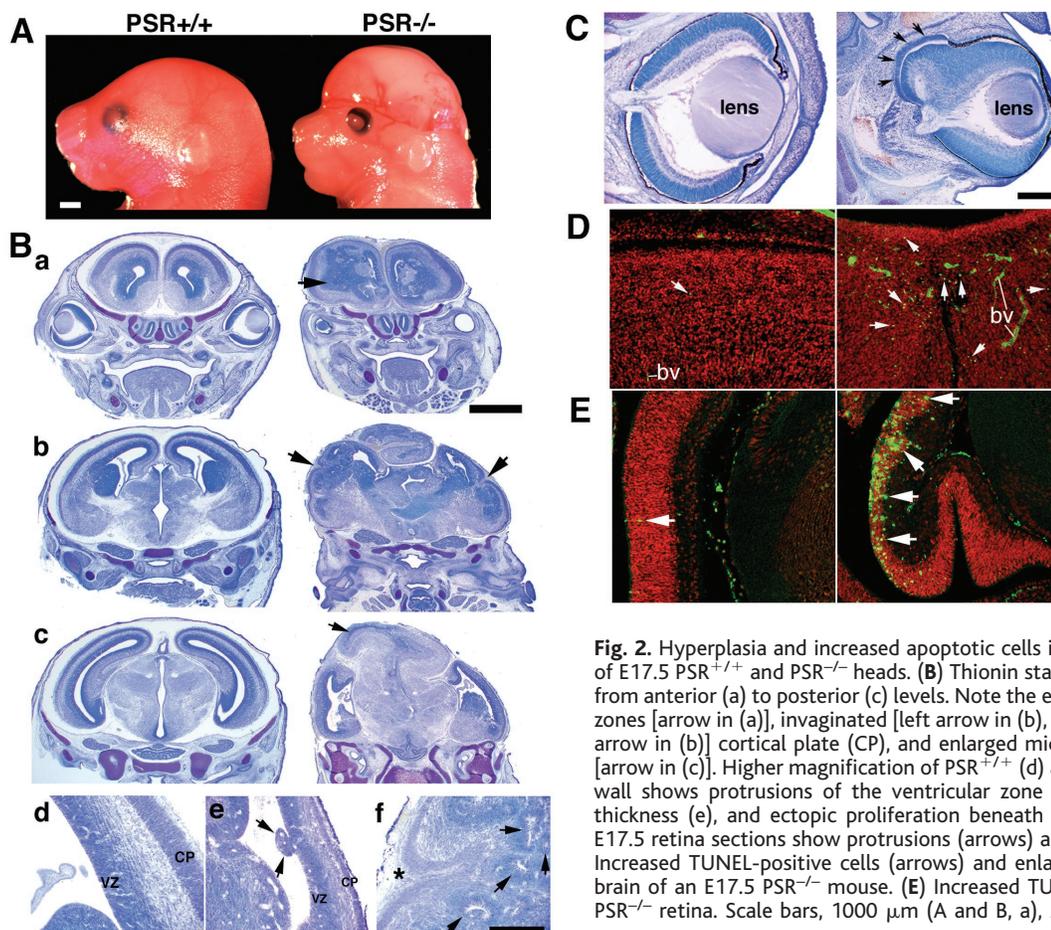
onic stem (ES) cells by homologous recombination (fig. S1C). Germline transmission of one targeted ES clone was confirmed by Southern blot analysis (fig. S1D), and the absence of PSR expression in homozygous mice was confirmed by Northern blot analysis (fig. S1E). PSR heterozygous mice appeared healthy and were comparable in all analyses to wild-type mice. Breeding of heterozygous mice, however, resulted in no PSR-deficient mice genotyped at postnatal day 8 (P8) (table S1). Viable PSR knockout mice could be identified at birth. PSR-deficient mice were unable to breathe, showed cyanotic skin color (Fig. 1A), and died within hours. Examination of lungs from these mice indicated that they were not fully expanded (Fig. 1B). Fetal lung development in mammals proceeds from a semisolid to a saccular organ capable of air exchange at birth. Histological analysis revealed that lumen formation in PSR-deficient lungs was severely impaired (Fig. 1C). Surfactant made by lung epithelial cells is essential for the creation of air space. Deficiency of surfactant protein results in postnatal respiratory failure (11); however, surfactant was present at seemingly normal amounts in the airways of PSR-deficient mice (Fig. 1C). Semiquantitative reverse transcription polymerase chain reaction

analysis and immunocytochemistry also revealed similar amounts of surfactant protein in PSR-deficient lungs (fig. S2). Thus, the death of PSR-deficient mice from impaired respiration is unlikely to be caused by defective surfactant production.

Lung morphogenesis is attributable in part to apoptosis and subsequent clearance of mesenchymal and epithelial cells (12–14). We used TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) to investigate cell apoptosis during lung development. Increased TUNEL-positive cells (~0.6% wild-type versus ~3.6% knockout cells) were noticed in the lungs of E17.5 PSR-deficient mice (Fig. 1D) (fig. S2). Costaining of TUNEL with a type II epithelial cell marker, SP-C, indicated that these apoptotic cells comprise both mesenchymal and epithelial cells (fig. S2). To study the fate of these apoptotic cells, we examined the ultrastructure of cells from P0 mice. In wild-type lung, most apoptotic cells were found engulfed (Fig. 1E) (fig. S2), but no phagocytosis of apoptotic cells was detectable in PSR-deficient lungs. Rather, a population of necrotic-like cells was observed. These cells, of both mesenchymal and epithelial origin, had semi-condensed nuclei and dilated organelles in-

cluding mitochondria and nuclear membranes (Fig. 1E) (fig. S2). Recruitment of neutrophils, a sign of inflammation associated with cell necrosis, was also evident in regions with lysed cells in PSR-deficient lungs (15). These observations suggest that the increased number of apoptotic cells in PSR-deficient lungs is likely due to failed clearance. Remaining apoptotic cells may undergo secondary necrosis that is associated with pulmonary dysfunction in these mice.

PSR-deficient mice were generated in close to Mendelian ratios at E15.5 and E17.5 (table S1), which suggests that PSR is dispensable for the survival of early-stage embryos. In addition to defective lung development, ~15% of PSR-deficient embryos manifested a severe brain malformation (Fig. 2A) characterized by exencephaly, disrupted forebrain proliferative zones, expanded midbrain, and a disrupted cortical plate (Fig. 2B) (fig. S3). We also noted altered morphology of the olfactory bulb, brainstem–spinal cord junction, and cerebellum (fig. S3). Notably, the eyes of E17.5 PSR-deficient mice show protrusions of the retinal neuroepithelium and smaller lenses (Fig. 2C). Enhanced proliferation within PSR-deficient retina and brain was consistently observed (fig. S3). Ectopic proliferative zones were in some instances highly immunopositive for the neuronal marker



**Fig. 2.** Hyperplasia and increased apoptotic cells in PSR-deficient CNS. (A) Phenotype of E17.5 PSR<sup>+/+</sup> and PSR<sup>-/-</sup> heads. (B) Thionin staining of E15.5 coronal brain sections from anterior (a) to posterior (c) levels. Note the expanded periventricular proliferative zones [arrow in (a)], invaginated [left arrow in (b), asterisk in (f)] and interrupted [right arrow in (b)] cortical plate (CP), and enlarged midbrain showing ectopic proliferation [arrow in (c)]. Higher magnification of PSR<sup>+/+</sup> (d) and PSR<sup>-/-</sup> [(e) and (f)] telencephalic wall shows protrusions of the ventricular zone (VZ) [arrows in (e)], decreased CP thickness (e), and ectopic proliferation beneath the CP [arrows in (f)]. (C) Coronal E17.5 retina sections show protrusions (arrows) and a smaller lens in PSR<sup>-/-</sup> mice. (D) Increased TUNEL-positive cells (arrows) and enlarged blood vessels (bv) in the mid-brain of an E17.5 PSR<sup>-/-</sup> mouse. (E) Increased TUNEL-positive cells (arrows) in E17.5 PSR<sup>-/-</sup> retina. Scale bars, 1000  $\mu$ m (A and B, a), 200  $\mu$ m (B, f), 100  $\mu$ m (C).

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TUJ1 (fig. S3), indicating that these zones contain abnormally migrated or prematurely differentiated neuronal cells. Thus, PSR appears to regulate the size of different proliferative cell populations during neuronal development.

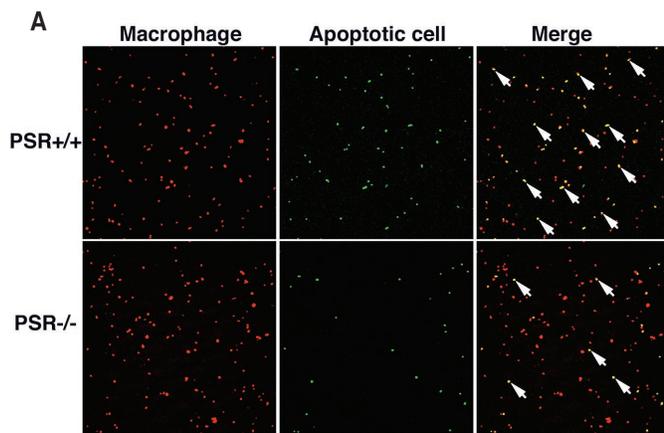
Apoptosis plays an essential role in eliminating overproduced progenitors during development of the central nervous system (CNS) (16, 17). Inactivation of the Apaf-1–caspase-9–caspase-3 apoptosome pathway in mice results in decreased cell apoptosis and hyperplasia of similar CNS structures that showed abnormalities in PSR-deficient mice (18–22). However, unlike mice deficient in the caspase pathway, which have decreased apoptosis, PSR-deficient mice at E15.5 to E17.5 showed increased TUNEL-positive cells within the midbrain (~0.9% wild-type versus ~6.8% knockout cells) (Fig. 2D) and retina (Fig. 2E). The affected midbrain also exhibited numerous pyknotic nuclei, increased active-caspase-3 immunostaining (15), and enlarged blood vessels, suggesting an inflammatory response to this region (Fig. 2D). The inflammatory response in PSR-deficient midbrain was confirmed by recruitment of F4/80-positive macrophages to this region (fig. S4). In addition, electron microscopy (EM) analysis of PSR-deficient midbrain showed defective engulfment of apoptotic cells (fig. S4). These data are consistent with a role for PSR in the removal of apoptotic cells in the CNS, lack of which also results in a hyperplastic phenotype.

To test phagocyte function in the absence of PSR, we performed adoptive transfer experiments to generate PSR-deficient macrophages. E14.5 fetal livers, which harbor hematopoietic stem cells, from both wild-type and knockout mice were injected into lethally irradiated recipient mice (B6.SJL-Ptprc strain). Because B6.SJL-Ptprc mice have a different hematopoietic marker from the donor mice (CD45.1 versus CD45.2), cells from the donor or the recipient could be distinguished. Four weeks after transfer, thioglycollate-elicited macrophages were isolated. Staining of the macrophages by an antibody to CD45.2 revealed that more than 90% of the cells were derived from donor mice (15). Apoptotic T cells were incubated with these macrophages and the engulfment was quantified. A 50% reduction of phagocytosis was detected in macrophages derived from PSR-deficient mice (Fig. 3, A and B). To test whether the defective engulfment in PSR-deficient macrophages reflects a defect in the specific recognition of PS, we performed a liposome competition experiment. PS liposomes, but not phosphatidylcholine (PC) liposomes, inhibited phagocytosis by PSR wild-type macrophages and had no effect on PSR-deficient macrophages (Fig. 3B). Hence, PSR has an essential role in the recognition of PS. In contrast, similar amounts of phagocytosis were observed when T cells opsonized with antibodies to Thy1.2, a protein present on all T cells, were used (fig. S5),

indicating that PSR-deficient macrophages do not display a general defect in phagocytosis. These results demonstrate that PSR is required for phagocytosis of apoptotic cells.

A number of molecules and surface receptors are implicated in phagocytosis of apoptotic cells in mammals (23, 24). Ablation of these molecules in mice, however, has resulted in either no deficiency or minor defects in embryonic development (25). In contrast, mice deficient in PSR manifested severe lung and brain phenotypes associated with increased numbers of non-phagocytosed apoptotic cells. Increased density of apoptotic cells in the malformed brains of PSR-deficient mice suggests that PSR may function in the removal of apoptotic cells. Yet the hyperplastic phenotype of such mice resembles that seen in mice deficient in the apoptosome pathway. However, the penetrance of the brain phenotype in the latter mice is greater than we have observed in PSR-deficient mice (~15% exhibit hyperplasia). It is not clear why this penetrance is lower. Because mice were generated on a mixed background (129 and C57BL/6), mixed genetic determinants may predispose knockout mice to this brain phenotype, as we have observed for caspase-3 knockout mice (26). Breeding these mice into a pure genetic background will be helpful in testing this hypothesis.

In any event, PSR-dependent phagocytosis may be important for killing cells, and failure of this process may be involved in human congenital brain malformations with defects in cell proliferation (for example, megalencephaly). In *Caenorhabditis elegans*, impaired phagocytosis can rescue cells from caspase-dependent apoptosis (27, 28). Therefore, phagocytes may induce and/or enhance apoptosis in cells destined to die. All newborn PSR-deficient mice suffered respiratory difficulties resembling those of respiratory distress syndrome (RDS) in human infants. Defects in surfactant protein expression are linked to some inherited RDSs (29) but were not observed in PSR-deficient mice. Impaired PSR-mediated phagocytosis of apoptotic cells may provide a mechanism underlying some forms of RDS. PSR is therefore essential for development of mouse lung and brain, underscoring the importance for removing apoptotic cells during mammalian development.



**Fig. 3.** Defective phagocytosis of apoptotic cells in PSR-deficient macrophages. **(A)** Macrophages (red) were cultured with apoptotic thymocytes (green). Engulfed apoptotic cells are indicated by arrows. **(B)** Quantification of phagocytosis of apoptotic cells by PSR<sup>+/+</sup> (wild-type, wt) and PSR<sup>-/-</sup> (knockout, ko) macrophages in the absence (untreated, UT) or presence of PS or PC liposomes.

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# Cell Corpse Engulfment Mediated by *C. elegans* Phosphatidylserine Receptor Through CED-5 and CED-12

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During apoptosis, phosphatidylserine, which is normally restricted to the inner leaflet of the plasma membrane, is exposed on the surface of apoptotic cells and has been suggested to act as an "eat-me" signal to trigger phagocytosis. It is unclear how phagocytes recognize phosphatidylserine. Recently, a putative phosphatidylserine receptor (PSR) was identified and proposed to mediate recognition of phosphatidylserine and phagocytosis. We report that *psr-1*, the *Caenorhabditis elegans* homolog of PSR, is important for cell corpse engulfment. In vitro PSR-1 binds preferentially phosphatidylserine or cells with exposed phosphatidylserine. In *C. elegans*, PSR-1 acts in the same cell corpse engulfment pathway mediated by intracellular signaling molecules CED-2 (homologous to the human Crkl protein), CED-5 (DOCK180), CED-10 (Rac GTPase), and CED-12 (ELMO), possibly through direct interaction with CED-5 and CED-12. Our findings suggest that PSR-1 is likely an upstream receptor for the signaling pathway containing CED-2, CED-5, CED-10, and CED-12 proteins and plays an important role in recognizing phosphatidylserine during phagocytosis.

involvement of PSR in recognizing PS and in removing apoptotic cells, we characterized the *C. elegans* PSR homolog, *psr-1*, which is defined by an open reading frame F29B9.4 and encodes a 400-amino acid protein with 56% sequence identity and 72% sequence similarity to the human PSR protein (fig. S1) (11). In an enzyme-linked immunosorbent assay (ELISA), recombinant PSR-1, produced and purified from *Escherichia coli*, preferentially bound PS over phosphatidylinositol (PI), phosphatidylethanolamine (PE), or phosphatidylcholine (PC) and displayed a binding preference to phospholipids similar to that of human PSR (Fig. 1A). Thus, PSR-1 appears to be a PS-specific binding protein. Human Jurkat T lymphocytes transiently transfected with worm PSR-1 bound to apoptotic Jurkat T cells or symmetric red blood cell

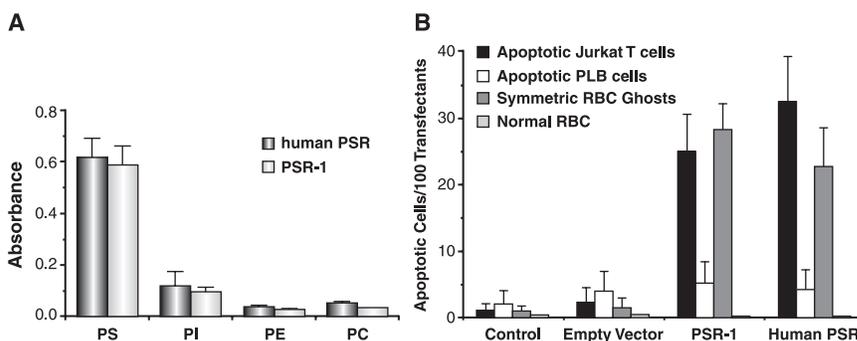
Although the important role of phosphatidylserine (PS) in presenting apoptotic cells for phagocytosis is well established (1–10), the

mechanism by which it is recognized by phagocytes to trigger the phagocytosis event remains elusive. To investigate the potential

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**Fig. 1.** Phosphatidylserine binding by *C. elegans* PSR-1. **(A)** Preferential binding of PS by recombinant PSR-1 and human PSR proteins in an ELISA assay. PSR-1 and human PSR proteins were expressed in *E. coli* and purified as described (13). Microtiter plates were coated with lipids as described (27). PSR-1 or human PSR (100 μg) was added to quadruplicate wells for each lipid and incubated overnight at 4°C. Bound protein was detected with monoclonal antibody 217G8E9; the binding of this antibody to PSR-1 was supported by equivalent absorbance results using an antibody to His<sub>6</sub> to detect the N-terminal polyhistidine tag on PSR-1 (28). Results represent the mean ± SEM of four separate experiments, with quadruplicate data points from each experiment. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine. **(B)** Human Jurkat T lymphocytes transiently transfected with PSR-1 bind to PS-expressing apoptotic cells and red blood cell ghosts. Jurkat cells were transfected with either the PSR-1- or the human PSR-expressing vector (13), then examined after 48 hours for their ability to bind to apoptotic Jurkat T cells (PS+), apoptotic PLB 985 cells (PS−) (12),



symmetric red blood cell (RBC) ghosts (PS+), and normal red blood cells (PS−). Binding was quantified by light microscopy. Binding experiments were performed on cells obtained from three separate transfections. Within each experiment, binding was assessed in triplicate. Data are expressed as the mean ± SEM. Transfection efficiency was 27.5 ± 5.6%.