

# The Presumptive Phosphatidylserine Receptor Is Dispensable for Innate Anti-inflammatory Recognition and Clearance of Apoptotic Cells\*

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The role of the presumptive phosphatidylserine receptor (PSR) in the recognition and engulfment of apoptotic cells, and the anti-inflammatory response they exert, has been of great interest. Genetic deficiency of PSR in the mouse is lethal perinatally, and results to date have been ambiguous with regard to the phagocytic and inflammatory phenotypes associated with that deficiency. Recently, we found that the specific functional recognition of apoptotic cells is a ubiquitous property of virtually all cell types, including mouse embryo fibroblasts, and reflects an innate immunity that discriminates live from effete cells. Taking advantage of this property of fibroblasts, we generated PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> fibroblast cell lines to examine definitively the involvement of PSR in apoptotic recognition and inflammatory modulation. Our data demonstrate that PSR-deficient cells are fully competent to recognize, engulf, and respond to apoptotic cells. Signal transduction in the responder cells, including the activation of Akt and Rac1, is unimpaired in the absence of PSR. We confirm as well that PSR is localized predominantly to the nucleus. However, it does not play a role in pro-inflammatory transcription or in the anti-inflammatory modulation of that transcriptional response triggered by apoptotic cells. We conclude that PSR is not involved generally in either specific innate recognition or engulfment of apoptotic cells.

Physiological cell death is a process whose purpose is the elimination of functionally inappropriate cells in a manner that does not elicit inflammation. The ability of apoptotic corpses to be cleared in a non-inflammatory manner by phagocytes is a consequence of their specific expression of determinants for recognition and modulation of pro-inflammatory responses. The acquisition of these apoptotic determinants is a gain-of-function common to all physiological cell deaths, without regard to suicidal stimulus, and conserved widely across species (1, 2).

Numerous cellular alterations associated with apoptotic cell death have been described, including plasma membrane reorganization asso-

ciated with blebbing (3), shrinkage, and the loss of membrane phospholipid asymmetry (4, 5). In particular, phosphatidylserine (PS),<sup>4</sup> an anionic phospholipid normally cloistered in the inner leaflet of the plasma membrane, is externalized during physiological cell death (5). It still remains to be determined what specific molecular events are responsible for the recognition of the effete cell.

The view that externalized PS serves as a ligand for macrophage recognition of apoptotic cells followed from studies demonstrating that similar changes target aged erythrocytes for clearance (6, 7) and gained support from observations that phospho-L-serine and PS vesicles could inhibit partially the interaction of dying nucleated cells with macrophages (5, 8, 9).

A presumptive cell surface PS-specific receptor (PSR) was identified molecularly following a screen for monoclonal antibodies whose binding to human macrophages was inhibited by PS-containing liposomes (10). The product of that screen, mAb 217, bound to cell surface determinants on macrophages and other cell types, notably excluding lymphoid cells. Significantly, mAb 217 triggered macrophages to release the anti-inflammatory cytokine TGF $\beta$ , further suggesting that mAb 217 engaged an apoptotic-like recognition mechanism (10).

Controversy regarding this presumptive receptor arose, however, when PSR was observed to localize to the nucleus in mammalian cells (11) as well as in *Hydra* (12). The role of PSR has been further clouded by the disparate results of three groups of investigators who independently generated mice with targeted disruptions of the PSR locus (13–15). While homozygous PSR disruptions result in perinatal lethality in each case, different effects on the phagocytosis of apoptotic cells have been reported for the three PSR deficiencies.

Li *et al.* (13) described a disruption beginning upstream of exon 1 (which includes the translational start site of the PSR gene product) and extending through exon 2, in a mixed 129  $\times$  C57BL/6 background, which leads to severe lung defects as well as less penetrant brain aberrations. Kunisaki *et al.* (14) generated a disruption, also starting upstream of exon 1 and extending into exon 3, in a chimeric 129  $\times$  C57BL/6 background, which results in defective erythroid differentiation and severe anemia. The disruption generated by Böse *et al.* (15) in a pure C57BL/6 background is limited to exons 1 and 2 and leads to

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<sup>4</sup> The abbreviations used are: PS, phosphatidylserine; CFDA, 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester; CMTMR, 5 (and 6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; GFP, green fluorescent protein; GST, glutathione S-transferase; MFG-E8, milk fat globule factor-E8; PSR, presumptive PS-specific receptor; RT, reverse transcriptase; IL-1 $\beta$ , interleukin 1 $\beta$ ; IL-6, interleukin 6; TGF $\beta$ , transforming growth factor- $\beta$ ; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; mAb, monoclonal antibody; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; CRIB, Cdc42/Rac interactive binding.

growth retardation, defects in embryonic lung, kidney, gut, and erythroid development, and, at low frequency, aberrant eye and brain development. Hemizygous PSR deficiency has no phenotype in any of these cases.

An increased number of apoptotic cells (with digested genomic DNA, identified by TUNEL (terminal deoxyribonucleotidyltransferase end labeling) staining) was observed by Li *et al.* (13) in the affected tissues of their PSR-deficient mice and attributed to the diminished engulfment of dead cells. Interestingly, Kunisaki *et al.* (14) also suggested that phagocytosis of apoptotic cells by macrophages was diminished during embryogenesis, based on immunohistochemical staining of fetal liver and thymus sections of their PSR<sup>-/-</sup> mice. Their data are confounded, however, by an apparent reduction in the number of tissue-resident macrophages (stained with F4/80 antibody; Ref. 16), hinting at another possible developmental impairment related to PSR deficiency. Li *et al.* (13) further explored engulfment *in vitro* with PSR-deficient macrophages generated by adoptive transfer of PSR<sup>-/-</sup> fetal liver into wild-type hosts. They reported a 50% overall reduction and a complete absence of PS-inhibitable engulfment by PSR-deficient elicited macrophages. In light of the observations of Kunisaki *et al.* (14), it is important to note that the differentiation and activation states of these PSR<sup>-/-</sup> and PSR<sup>+/+</sup> macrophages were not compared; previous work has suggested that PS-inhibitable engulfment pertains particularly to activated macrophages (8, 9). In marked contrast to the results of Li *et al.* (13) and Kunisaki *et al.* (14), extensive histological analysis of numerous tissues by Böse *et al.* (15) revealed no defects in the phagocytosis of apoptotic (TUNEL<sup>+</sup>) cells. Their *in vitro* phagocytosis studies with fetal liver-derived macrophages (differentiated in culture) also showed no engulfment defect of PSR-deficient macrophages. Of great significance, Böse *et al.* (15) reported that the ability of apoptotic cells to trigger an anti-inflammatory response in engulfing macrophages (both the inhibition of secretion of pro-inflammatory cytokines, such as TNF $\alpha$ , and the induction of anti-inflammatory cytokines, such as TGF $\beta$  and IL-10) was unimpaired in the absence of PSR expression.

Our studies of the process of apoptotic cell clearance have demonstrated that the ability of apoptotic corpses to be engulfed specifically and in a non-inflammatory manner by macrophages and other phagocytes is a consequence of a process of specific recognition and modulation of pro-inflammatory phagocyte responses (1). The modulatory effect of the apoptotic corpse is manifest as an immediate-early inhibition of pro-inflammatory cytokine gene transcription within the responding phagocyte with which it interacts, and is exerted upon binding, independent of subsequent engulfment or soluble factor involvement (2). Cells that die pathologically (that is, necrotic corpses) also are recognized by phagocytes but do not down-regulate inflammatory responses. The recognition of these two classes of native dying cells occurs via distinct and non-competing mechanisms (1).

Importantly, non-professional phagocytes are fully capable of non-inflammatory recognition and clearance of apoptotic cells.<sup>5</sup> Indeed, we have found that the specific functional recognition of apoptotic cells is a ubiquitous property of virtually all cell types, including non-phagocytic lymphocytes, and reflects an innate immunity that discriminates live from effete cells without regard to self (2).<sup>5</sup> In particular, the ability of fibroblastic cells to respond to apoptotic corpses permits mouse embryo fibroblasts established from animals with targeted disruptions of genes of interest (including essential genes) to be used in the evaluation of genetic contributions to apoptotic recognition and response without the generation or selection of particular cell populations.

We have applied this analysis to embryo fibroblasts that harbor a disruption of the PSR locus. Our data, which demonstrate that PSR-deficient cells are fully competent to recognize apoptotic cells, definitively exclude a general role for PSR in apoptotic recognition and inflammatory modulation.

## EXPERIMENTAL PROCEDURES

**Cells and Death Induction**—Immortalized murine fibroblast cell lines were derived from mouse embryo fibroblasts (MEFs) following the 3T3 protocol of Todaro and Green (17). Briefly, MEFs were cultured at 37 °C in a humidified, 5% (v/v) CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium with 4.5 g/liter glucose (Mediatech, Herndon, VA) supplemented with fetal bovine serum (10% v/v; HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 50  $\mu$ M 2-mercaptoethanol, replating at  $3 \times 10^5$ /60-mm diameter dish every 3 days. Immortalized cell lines were established from cells that grew from cultures that had become senescent.

NIH 3T3 cells were maintained similarly. 293T human transformed kidney epithelial cells were grown in the same medium without 2-mercaptoethanol, and DO11.10 murine T hybridoma and CEM human T lymphoblastoid cells were grown in RPMI 1640 medium (Mediatech) supplemented with heat inactivated fetal bovine serum (10% v/v), 2 mM L-glutamine, and 50  $\mu$ M 2-mercaptoethanol.

Physiological cell death (apoptosis) was induced by treatment of target cells with the macromolecular synthesis inhibitor actinomycin D (200 ng/ml, 12 h; Ref. 18) or with UV-B irradiation (20 mJ/cm<sup>2</sup>). Cells were killed pathologically (necrotic death) by incubation at 55 °C for 20 min (until trypan blue uptake indicated compromise of membrane integrity; Ref. 1). In all cases, target cells (viable, apoptotic, and necrotic) were washed twice in PBS or complete medium before addition to experimental cultures.

**Reverse Transcriptase (RT)-PCR Analysis**—PSR expression on the level of transcripts was evaluated by RT-PCR analysis. Total cellular RNA was isolated using TRIzol reagent (Invitrogen). cDNA synthesis and PCR were performed sequentially using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen). For the PSR-specific reaction, the forward primer was 5'-CAAGACGGTAAGAGGGGA-GACC-3' (nucleotides 1086–1106, within exon 4), and the reverse primer was 5'-GTCACCTGGAGGAGCTGCG-3' (complementary to nucleotides 1402–1384, within exon 6), yielding a product of 276 bp. As a control for RNA integrity, transcripts of constitutively expressed glyceraldehyde-3-phosphate dehydrogenase were assessed in parallel, using forward (5'-CCATGGAGAAGGCTGGGG-3') and reverse (5'-CAAAGTTGTCATGGATGACC-3') primers, generating a 188-bp product from 5' end of the mRNA.

**Phagocytosis Assay**—Phagocytosis by 3T3 fibroblasts was assessed as previously described for macrophages (2). Target cells were labeled green with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA; 0.2  $\mu$ M; Molecular Probes, Eugene, OR) and were then induced to undergo cell death, killed by heat treatment, or left untreated. 3T3 cells were labeled red with 5 (and 6)-((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine (CMTMR; 10  $\mu$ M; Molecular Probes). In all cases, cells were labeled on the day preceding the experiment and cultured in serum-containing medium overnight to eliminate unbound label. Labeled 3T3 cells were co-cultured with the apoptotic, necrotic, or viable target cells for 30 min at 37 °C. Cells were harvested with PBS supplemented with 0.4 mM Na<sub>2</sub>EDTA and analyzed cytofluorimetrically on a FACS Caliber instrument (BD Biosciences). Cells with 3T3-like scatter properties that were both CMTMR-positive ( $\lambda_{Ex}$  = 488 nm,  $\lambda_{Em}$  = 610 nm  $\pm$  15 nm) and CFDA-positive ( $\lambda_{Ex}$  = 488 nm;

<sup>5</sup> M. Cvetanovic, J. E. Mitchell, V. Patel, B. S. Avner, Y. Su, P. T. van der Saag, P. L. Witte, S. Fiore, J. S. Levine, and D. S. Ucker, submitted for publication.

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$\lambda_{Em} = 530 \pm 15$  nm) represented 3T3 cells that had engulfed targets. Engulfment is calculated as the fraction of double-positive 3T3 fibroblasts (all CMTMR-positive cells that also are CFDA-positive). Targets that are bound but not engulfed are disrupted and do not remain adherent during the analysis.

Phagocytosis by transfected 293T cells was assayed similarly, employing a green fluorescent protein (GFP) marker expressed from a bicistronic vector to label the phagocytes green in place of CFDA. 293T cells were transfected with pIRES-2-PSR, pCX- $\beta_5$  (which includes the same cytomegalovirus promoter/internal ribosome entry site (IRES) structure), or empty pIRES-2 vector (19). Target cells were labeled red with PKH26-GL red fluorescent cell linker kit (Sigma). 48 h after transfection, the 293T cells were co-cultured with labeled apoptotic cells for 2 h at 37 °C and analyzed as above.

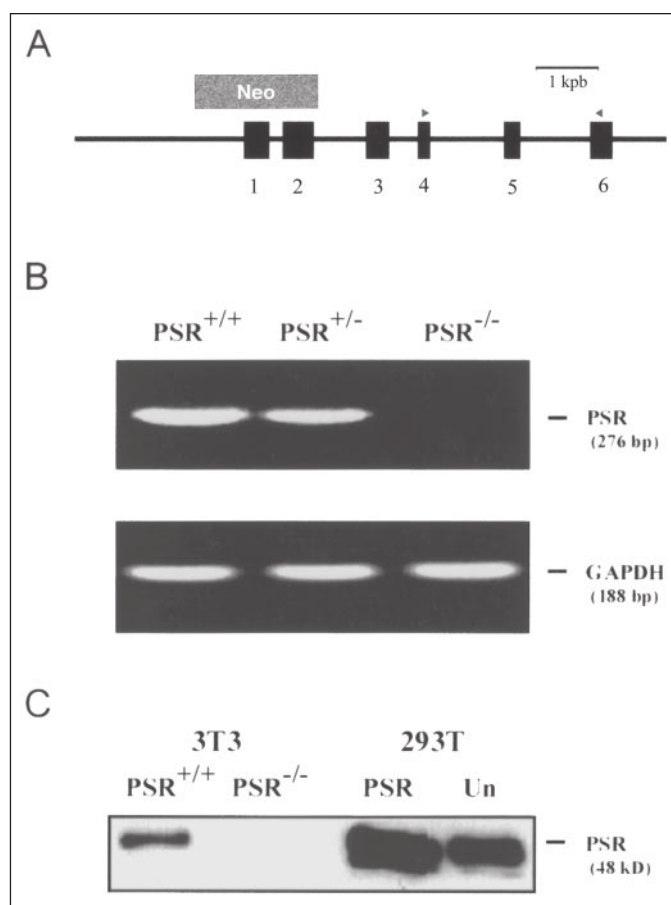
**Transfections and Luciferase Assays**—Apoptotic modulation of NF $\kappa$ B-dependent transcription was assessed using a dual luciferase strategy, as described previously (2). We found that routine transfection protocols for 3T3 cells triggered high levels of cell death and NF $\kappa$ B activation (“transfection stress”). A transfection protocol that was reasonably efficient (~40% viable cell transfection, as measured in parallel with farnesylated GFP as a transfection marker; Ref. 20) and minimally stressful (*i.e.* low spontaneous NF $\kappa$ B activation) was selected.

3T3 cells were transfected using the MEF1 Nucleofector Kit (AMAXA Biosystems, Gaithersburg, MD), with “MEF1 Nucleofector Solution 1” and a machine setting of “A-23.”  $2 \times 10^6$  3T3 fibroblasts were transfected with 4.5  $\mu$ g of pNF $\kappa$ B-Luc, a plasmid containing the firefly (*Photinus pyralis*) luciferase gene, the expression of which is driven by a basal transcriptional promoter linked to four copies of the  $\kappa$ B motif (Clontech Laboratories; Palo Alto, CA), together with 0.5  $\mu$ g of pRL-SV40, a *Renilla* (sea pansy; *Renilla reniformis*) luciferase control vector, the constant expression of which is dependent on the SV40 early enhancer/promoter region (Promega, Madison, WI). Transfected cells were cultured overnight in 100-mm diameter dishes and replated the following day into 24-well plates at  $1 \times 10^5$  cells/2 ml/well. After culturing a further 24 h, transfected cells were incubated without or with the indicated target cells (at a target cell: fibroblast ratio of 10:1) and/or IL-1 $\beta$  (5 ng/ml; R&D Systems; Minneapolis, MN) or TNF $\alpha$  (10 ng/ml; R&D Systems) for 12 h.

Cell extracts were prepared, and luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) in an FB12 Luminometer (Zylyx, Oak Ridge, TN). Each condition was repeated in triplicate wells, and the luciferase activities in cells from each well were determined independently. The firefly luciferase activity in each sample was normalized with respect to the internal *Renilla* luciferase activity, and the relative level of normalized firefly luciferase activity compared with the activity in an untreated population was taken as a measure of NF $\kappa$ B-dependent transcriptional activity.

For other studies involving transfection, routine methods were employed. NIH 3T3 cells were transfected with Lipofectamine 2000 Transfection Reagent (Qiagen, Valencia, CA), and 293T cells were transfected using Effectene Transfection Reagent (Qiagen).

**Intracellular Immunostaining**— $5 \times 10^4$  NIH 3T3 cells were plated on poly-D-lysine-coated cover slips and transfected with epitope-tagged PSR constructs (either N-terminal HA-PSR in pRK or C-terminal PSR-V5-His in pcDNA3.1). 24 h post-transfection, the cells were fixed with paraformaldehyde (5% in PBS). The cells then were permeabilized with Triton X-100 (0.1% in PBS) for 5 min and incubated for 1 h with anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-V5 (Invitrogen) antibodies (diluted in PBS + 0.1% gelatin). After four washes (5 min each) in PBS + 0.1% gelatin, the cells were stained with fluorescein

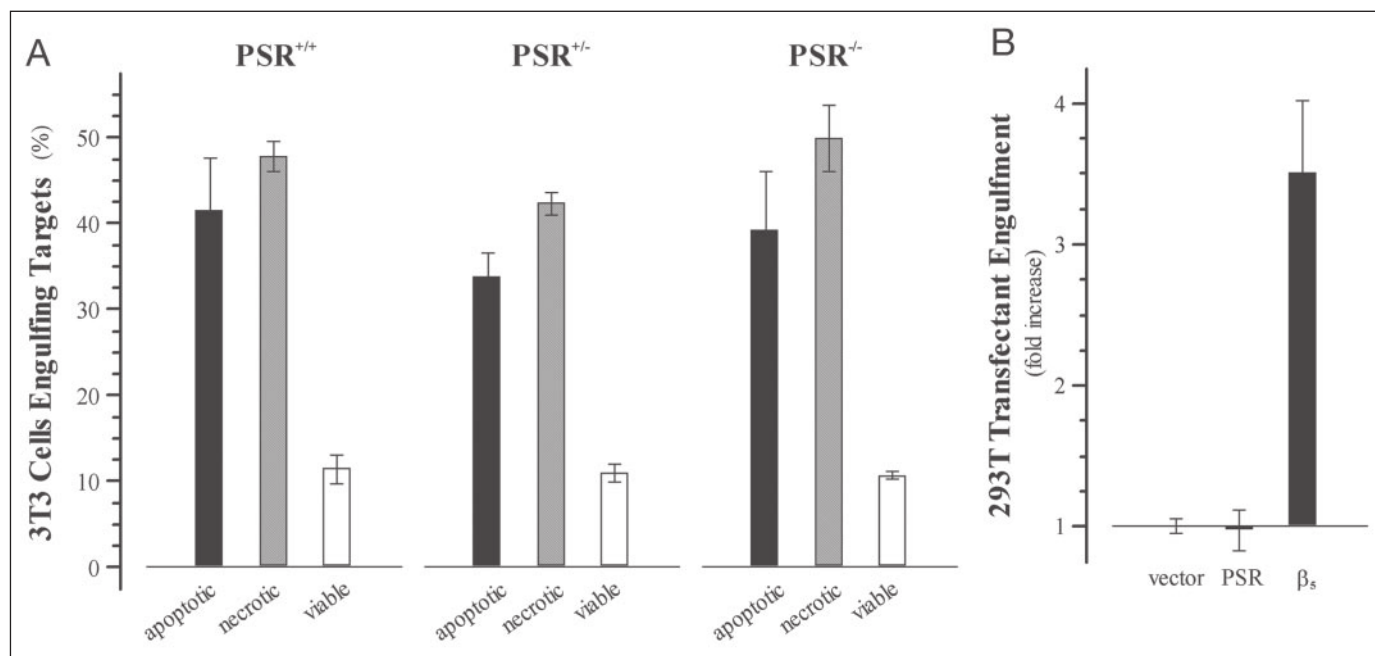


**FIGURE 1. Characterization of immortalized PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> murine 3T3 fibroblasts.** A, physical map of the murine PSR locus, which is comprised of six exons (filled boxes). The open reading frame for PSR begins in exon 1 and ends in exon 6. The PSR disruption generated by Li *et al.* (13) involves a selectable Neomycin resistance insertion spanning exons 1 and 2, indicated as a stippled box. Sites in exons 4 and 6 of PCR primers used for the analysis of PSR transcripts are marked as triangles. B, RT-PCR analysis of PSR expression in PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> 3T3 fibroblasts. Analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts was performed in parallel as a control for RNA integrity. C, immunoblot analysis of PSR expression in PSR<sup>+/+</sup> and PSR<sup>-/-</sup> 3T3 fibroblasts and in untransfected (Un) 293T cells and cells transfected with an HA-tagged PSR construct.

isothiocyanate-conjugated anti-rabbit IgG antibody and rhodamine-conjugated phalloidin (Molecular Probes). After several further washes, the coverslips were dried and mounted with mounting medium (Prolong Antifade Kit; Molecular Probes). The cells were visualized by epifluorescence microscopy.

**Cellular Extract Preparation and Immunoblot Analysis**—PSR expression was assessed in unmanipulated 3T3 cells by immunoblotting. Activation of Akt and inhibition of extracellular signal-regulated kinases 1 and 2 (ERK1/2) were assessed in 3T3 cells cultured overnight in serum-free medium and left unstimulated or stimulated for 15 min with a 5-fold excess of apoptotic DO11.10 cells (the apoptotic cells, which had been cultured under serum-free conditions, were centrifuged briefly onto the adherent 3T3 cells to initiate the interaction) and/or subsequent stimulation with epidermal growth factor (10 nM; Calbiochem). After washing, cell extracts were prepared from the adherent 3T3 cells. Cells were lysed in lysis buffer 1 (150 mM NaCl, 50 mM HEPES (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 200  $\mu$ M orthovanadate). Lysates were centrifuged at 10,000  $\times$  g for 10 min at 4 °C and the supernatants stored at -70 °C.





**FIGURE 2. PSR is dispensable for engulfment of apoptotic cells.** *A*, PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> murine 3T3 fibroblasts were fluorescently labeled red with CMTMR and apoptotic, necrotic, and viable DO11.10 murine T cell targets were prelabeled green with CFDA. 3T3 cells were incubated with apoptotic, necrotic, or viable targets at a target to 3T3 cell ratio of 10:1, for 30 min at 37 °C. Engulfing 3T3 cells, identified cytofluorimetrically as CMTMR- and CFDA-positive, were quantified. *B*, 293T cells, transfected with a vector driving expression of GFP alone or together with PSR or β<sub>5</sub> integrin, were cultured with apoptotic CEM human lymphoblastoid cells that had been labeled red with PKH26-GL, at a target to 293T cell ratio of 10:1. After 2 h at 37 °C, engulfing 293T cells were identified cytofluorimetrically as GFP- and PKH26-GL-positive and quantified. Results with apoptotic targets are represented as black bars, with necrotic targets as hatched bars and with viable cells as unshaded bars, and are expressed as the mean (± S.E.) of triplicate determinations.

Protein samples (20 μg each, determined by the bicinchoninic acid protein assay; Pierce) were boiled in 5× sample buffer, run on 12% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Blots were blocked with 5% dry milk in PBS before probing with a phospho-Akt(Thr<sup>308</sup>)-specific rabbit antiserum (Cell Signaling, Beverly, MA), an affinity-purified phospho-ERK1/2 (Thr<sup>183</sup>/Tyr<sup>185</sup>)-specific rabbit antibody (Promega), or an affinity-purified PSR-specific rabbit antibody (specific for amino acids 363–381; Abcam, Cambridge, MA). Following incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase, immunoreactive bands were visualized by the luminol reaction (ECLplus; Amersham Biosciences). Equivalent loading of protein samples was monitored by Ponceau S staining (0.25% (w/v; Sigma) in 0.1% acetic acid; 5 min) of blotted proteins.

**Rac1 Pull-down Assay**—The level of GTP-bound Rac1 was determined by the GST-PAK CRIB “pull-down” assay as described previously (21). In brief, 5 × 10<sup>5</sup> 3T3 fibroblasts were plated for 2 h on 60-mm diameter dishes that had been coated previously with MFG-E8 or bovine serum albumin (10 μg/ml). The cells then were lysed for 10 min in lysis buffer 2 (50 mM Tris (pH 7.2), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 500 mM NaCl, and 10 mM MgCl<sub>2</sub>, plus protease inhibitors). 500 μg of each cell lysate was incubated for 1 h with glutathione-agarose beads coated with a bacterially expressed fusion protein of glutathione S-transferase (GST) with the GTPase binding domain (CRIB domain; amino acid residue 56–272) of the human PAK kinase, a downstream effector molecule for Rac1, which specifically binds the activated, GTP-loaded form of Rac1. The beads then were washed four times in wash buffer (50 mM Tris (pH 7.2), 1% (v/v) Triton X-100, 150 mM NaCl, and 10 mM MgCl<sub>2</sub>, plus protease inhibitors). Active Rac1, precipitating with beads, and total Rac1, in the starting lysates, were quantified by densitometry following immunoblotting with a Rac1 antibody (Upstate Biotechnology, Waltham, MA).

## RESULTS

**Immortalized 3T3 Cell Lines from PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> Mouse Embryo Fibroblasts**—The role of PSR in the non-inflammatory clearance of apoptotic cells remains unresolved. Taking advantage of the ability of non-professional phagocytes to recognize, engulf, and respond to apoptotic cells specifically,<sup>5</sup> we established immortalized 3T3 fibroblast cell lines from PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> mouse embryo fibroblasts to test the role of PSR in these processes. The immortalized cell lines were derived from (129 × C57BL/6) embryo fibroblasts taken at day E14.5, prior to manifestations of lethality associated with PSR deficiency (13), following the 3T3 protocol of Todaro and Green (17). We chose to examine cells with the targeted PSR locus of Li *et al.* (Fig. 1A; Ref. 13), because the best genetic evidence implicating PSR in apoptotic clearance is derived from studies of mice harboring that disruption.

PSR expression in these cells was evaluated by RT-PCR analysis of PSR transcripts (Fig. 1B) and by immunoblotting with a PSR-specific antibody (Fig. 1C). These tests confirmed both the presence of PSR expression in wild-type cells and in PSR<sup>+/-</sup> heterozygotes, at roughly equivalent levels, and its absence in PSR<sup>-/-</sup> fibroblasts. These results are consistent with the genotypes of those cells, with the reported expression of PSR in fibroblastic cells (10), and with the lack of phenotypic defects associated with PSR hemizygoty (13–15).

**Phagocytosis of Apoptotic and Necrotic Corpses Is Unimpaired in the Absence of PSR**—We asked whether uptake of apoptotic target cells by 3T3 fibroblasts occurred normally in the absence of PSR expression. We tested the ability of PSR-deficient cells to engulf apoptotic targets, as well as necrotic and viable cells, and compared their activity with that of wild-type and PSR-heterozygous fibroblasts. As shown in Fig. 2A, the fibroblasts effectively discriminated viable from dead target cells and were highly phagocytic for both apoptotic and necrotic cells (as we have

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seen previously).<sup>5</sup> Importantly, they were fully competent for apoptotic phagocytosis regardless of PSR expression.

That the absence of PSR expression has no effect on target cell uptake indicates clearly that PSR serves no essential role in phagocytosis. This is surprising, because defects in engulfment have been attributed to PSR deficiency (13, 14). Furthermore, recent data have suggested that PS is involved in triggering the phagocytosis of already bound targets (22). By extension, the receptor for PS would be expected to be involved in engulfment as well.

We explored the potential involvement of PSR in engulfment in an independent and complementary manner. Engulfing activity has been

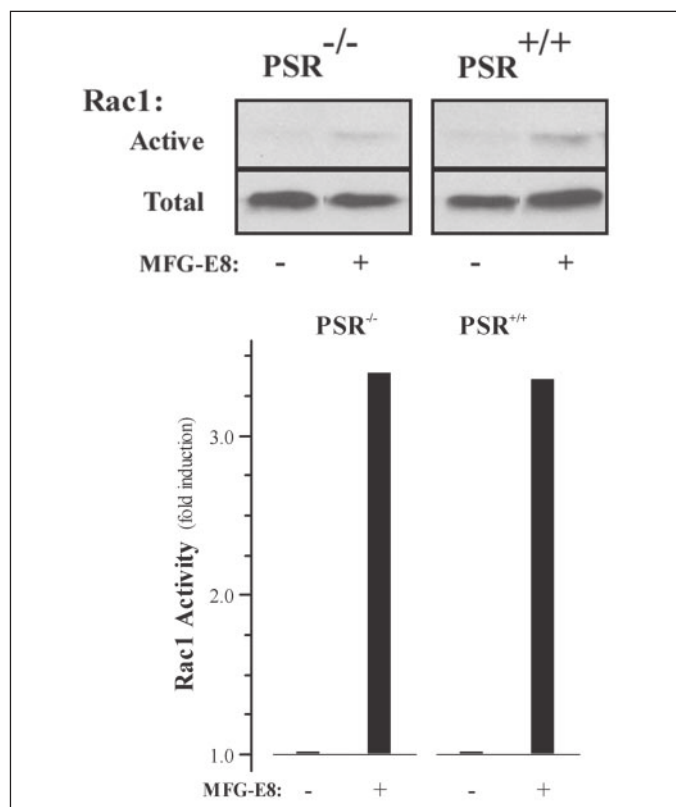
shown to be enhanced by overexpression in the phagocyte of molecules involved normally in the process of engulfment; this is exemplified with  $\alpha_v\beta_5$  integrin, for instance (19). We transfected 293T cells with PSR to test whether overexpression would enhance phagocytosis; expression of both endogenous and epitope-tagged exogenous PSR is evident by immunoblot analysis (Fig. 1C). In contrast to  $\alpha_v\beta_5$  integrin overexpressed at a comparable level, however, the overexpression of PSR did not enhance phagocytic activity (Fig. 2B and data not shown). Together, these data indicate that neither deletion nor overexpression of PSR alters the extent of apoptotic cell engulfment and suggest that PSR is neither essential nor functionally involved in the process of engulfment of apoptotic cells.

**Rac1 Activation following Apoptotic Cell Interaction Is Unimpaired in the Absence of PSR**—The identities of some of the molecules involved in the clearance of apoptotic cells have been revealed most insightfully from studies in *Caenorhabditis elegans*, where engulfment has been shown to involve the products of at least seven genes. These gene products function in redundant pathways for the clearance of dead cells (23), and their orthologues have been implicated in the clearance of dead cells in mammals (24). Significantly, these pathways converge to activate the small GTPase Rac1, the mammalian Ced10 equivalent, involved in cytoskeletal rearrangement and process extension (25, 26). The *C. elegans* PSR orthologue has been proposed to act in this same pathway (27).

We tested whether PSR contributes to pathways leading to Rac1 activation in mammalian cells. The well characterized CrkII-DOCK180-Rac1 post-receptor intracellular signaling module can be initiated by engaging  $\alpha_v\beta_5$  integrin for the phagocytosis of apoptotic cells (19). The soluble glycoprotein milk fat globule factor-E8 (MFG-E8), which has been implicated as a putative tethering molecule involved in the process of apoptotic cell clearance (28), acts as a ligand of  $\alpha_v\beta_5$  integrin to activate Rac1 (21).

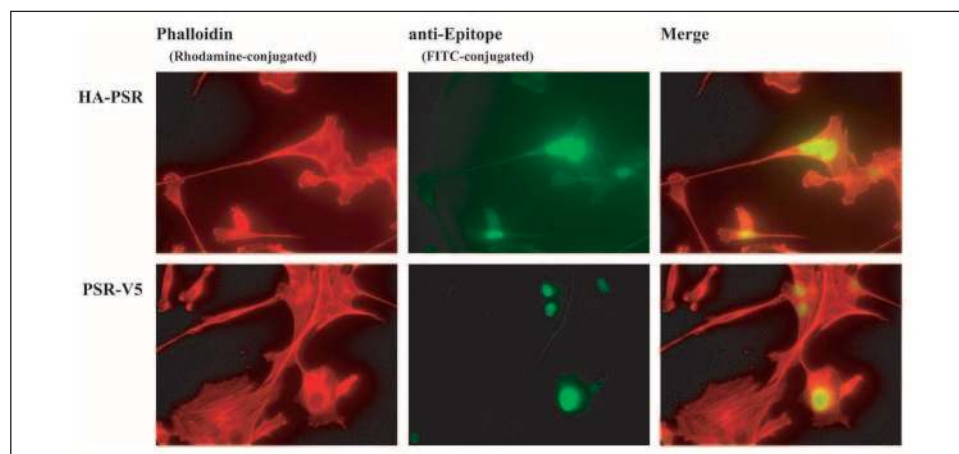
We explored the ability of MFG-E8 to activate Rac1 in PSR-deficient fibroblasts. The level of GTP-bound Rac1 was determined by a direct GST-PAK CRIB pull-down assay, as previously described (21). The data in Fig. 3 reveal that Rac1 activation ensued normally in the absence of PSR expression, further suggesting that normal Rho-GTPase signaling as well as phagocytosis occur in the absence of PSR expression.

**Specific Transcriptional Modulation Is Exerted Normally by Apoptotic Cells in the Absence of PSR**—Driscoll and colleagues (29) have demonstrated that the Rac1-dependent engulfment pathways in *C. elegans* are utilized for the clearance of necrotic as well as apoptotic targets, and we have shown that apoptotic and necrotic cells are recognized via distinct and non-competing mechanisms by mammalian cells

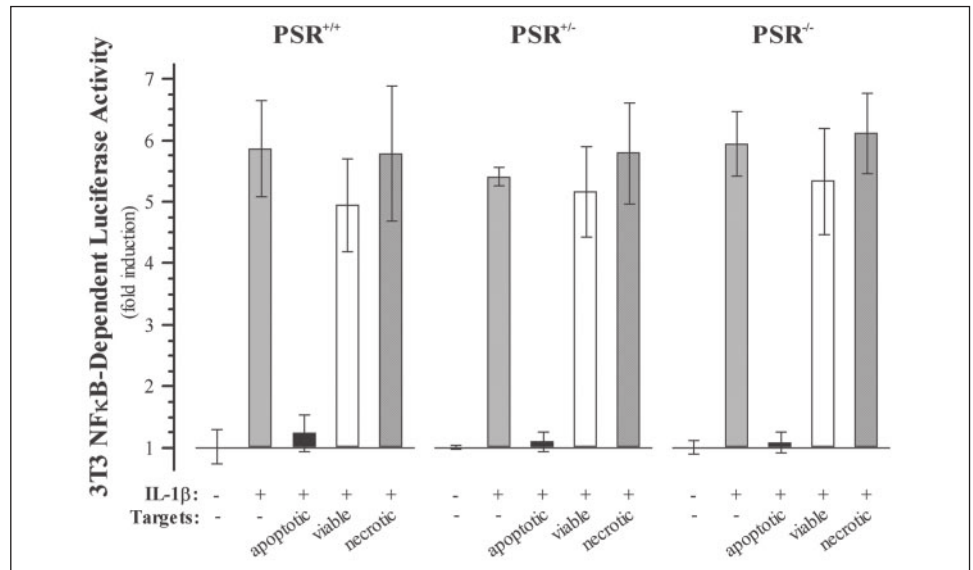


**FIGURE 3. PSR is dispensable for signal transduction triggered by apoptotic cell recognition leading to Rac1 activation.** PSR<sup>+/+</sup> and PSR<sup>-/-</sup> 3T3 fibroblasts were left unstimulated or stimulated for 2 h with MFG-E8. Active Rac1 in cell lysates was captured by GST-PAK CRIB pull-down and quantified following immunoblotting. Total Rac1 levels in cell lysates also were assessed by immunoblotting. The extents of stimulated Rac1 activation, normalized to untreated cells, are represented graphically in the lower panel.

**FIGURE 4. PSR localizes to the nucleus.** Epitope-tagged PSR was visualized in transfected 3T3 fibroblasts following fixation and permeabilization. Cells expressing N-terminal HA-tagged or C-terminal V5-tagged constructs were stained with anti-HA or anti-V5 antibodies, respectively, together with a fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody. Filamentous actin was stained with rhodamine-conjugated phalloidin. Stained cells were visualized by epifluorescence microscopy.



**FIGURE 5. PSR is dispensable for transcriptional modulation triggered by apoptotic cell recognition.** PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> murine 3T3 fibroblasts were transiently transfected with NFκB-Luc and an NFκB-independent *Renilla* luciferase normalization control vector, and luciferase activities were measured after incubation with IL-1β (5 ng/ml) and/or apoptotic, viable, or necrotic DO11.10 targets. 48 h after transfection, cells were incubated as indicated without or with IL-1β (5 ng/ml) and/or apoptotic, viable, or necrotic DO11.10 targets (at a target to responder ratio of 10:1). Cell extracts were prepared after 12 h, and luciferase activities were measured. Data are presented as normalized luciferase activities in treated 3T3 fibroblasts relative to the uninduced control population ("fold induction") and are the mean (± S.E.) of triplicate determinations. Results without target cells are represented as shaded bars, with apoptotic targets as black bars, with necrotic targets as hatched bars, and with viable targets as unshaded bars.



(1). In this context, phagocytosis and the activation of Rac1 may not be revealing of the potential involvement of PSR in a specific apoptotic recognition and clearance process.

On the other hand, the ability to trigger clearance without inflammation is the uniquely defining property of the apoptotic process. We have shown that the modulation of inflammatory responsiveness associated with apoptotic cell clearance is exerted on the level of pro-inflammatory cytokine gene transcription within the responding phagocyte. Apoptotic modulation of phagocyte transcription appears to be effected through a novel innate regulatory pathway and represents the earliest specific readout of apoptotic cell recognition (2).

Previous work indicating that PSR contains a jumoni-like (jmiC) chromatin binding motif (30) and localizes to the cell nucleus (11) allows the obvious speculation that it could be a regulator of inflammatory transcription linked to anti-inflammatory apoptotic recognition.

Indeed, we corroborated the nuclear localization of PSR by immunocytological staining. Epitope-tagged PSR, expressed in 3T3 fibroblasts and visualized with fluorescein isothiocyanate-conjugated epitope probes (Fig. 4), appeared exclusively in the nucleus. This localization, distinct from the rhodamine-conjugated phalloidin staining of cytoskeletal filamentous actin that marks the cytoplasm, is independent of the particular epitope tag and confirms the intrinsic nuclear localization of PSR.

A reporter of NFκB-dependent transcription reliably reveals the modulatory effect of apoptotic cells (2).<sup>5</sup> We transiently transfected the 3T3 fibroblasts with pNFκB-Luc, a plasmid containing the firefly luciferase gene, the expression of which is driven by a basal transcriptional promoter linked to four copies of the κB motif. Fibroblasts were co-transfected with a constitutive (NFκB-independent) *Renilla* luciferase control vector, which served as an internal normalization control. Following transfection, firefly and *Renilla* luciferase activities were measured in fibroblasts treated with combinations of NFκB stimuli and apoptotic target cells.

TNFα and IL-1β both were effective at stimulating NFκB-dependent transcription in the 3T3 fibroblasts; IL-1β was dependably better as a stimulus (Fig. 5 and data not shown). Note that it was necessary to adjust conditions for transfection of these cells so that high levels of NFκB-dependent transcription were not induced by the stress of the transfection procedure (see "Experimental Procedures"). We found no significant differences in the magnitude of NFκB-dependent responsiveness

among the 3T3 fibroblasts as a function of PSR expression (Fig. 5 and data not shown).

Recognition by 3T3 fibroblasts of apoptotic cells led to specific and potent transcriptional modulation. The robust NFκB-dependent response of fibroblasts stimulated by treatment with IL-1β was abrogated when those cells were incubated with apoptotic targets (Fig. 5). Apoptotic cell interactions did not lead to a global repression of fibroblast transcription, however; expression of the co-transfected *Renilla* luciferase control vector was unaffected by apoptotic targets, for instance (see Ref. 2). Viable and necrotic cells did not exert such modulation, consistent with our previous results (1, 2). Significantly, PSR expression had no effect upon apoptotic recognition and modulation (Fig. 5).

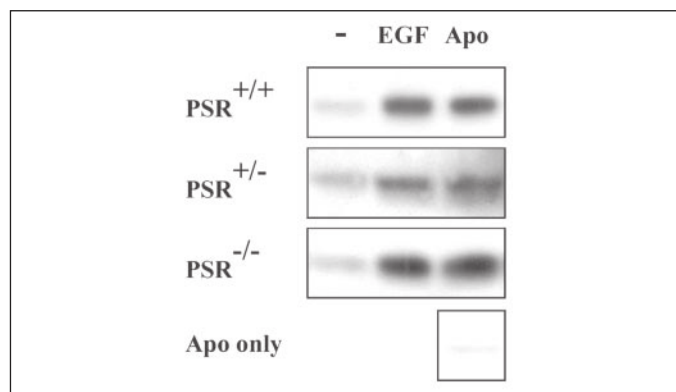
Apoptotic modulation also was observed when NFκB-dependent transcription was triggered with TNFα (data not shown); as we have noted previously, apoptotic modulation occurs independent of the particular stimulus (2). Correspondingly, we also observed that the IL-1β-stimulated secretion of IL-6 from 3T3 fibroblasts was attenuated specifically by their interaction with apoptotic targets, regardless of PSR expression (data not shown).<sup>5</sup>

*Specific Signaling Events following Apoptotic Cell Interaction Are Unimpaired in the Absence of PSR*—Novel signaling events, triggered within macrophages and non-professional phagocytes by their recognition of apoptotic cells, have been described recently that are associated with enhanced phagocyte survival and correlate with the modulation of inflammatory transcription (31, 32).<sup>5</sup> Among these are the activation of Akt, a protein kinase associated with death-sparing function, and the inhibition of ERK1/2.

We asked whether PSR affects these signaling responses. Activation of Akt, indicated by its specific phosphorylation on Thr<sup>308</sup>, was assessed by immunoblot analysis with a phospho-Akt-specific antibody. As shown in Fig. 6, 3T3 cells cultured overnight in the absence of serum contained little activated Akt, while stimulation of those cells with epidermal growth factor for 15 min triggered robust Akt activation. Incubation with apoptotic cells similarly triggered rapid and extensive Akt activation, and this response occurred in 3T3 cells regardless of their expression of PSR. The inhibition of ERK1/2 phosphorylation at its catalytic core (on Thr<sup>183</sup> and Tyr<sup>185</sup>) also was triggered normally following the interaction with apoptotic targets, irrespective of PSR expression (data not shown). These data extend our findings to demonstrate that all



## PSR Is Dispensable for Apoptotic Cell Recognition



**FIGURE 6. PSR is dispensable for signal transduction triggered by apoptotic cell recognition leading to Akt activation.** Activation of Akt was assessed in PSR<sup>+/+</sup>, PSR<sup>+/-</sup>, and PSR<sup>-/-</sup> 3T3 cells cultured overnight in serum-free medium and left unstimulated (-) or stimulated for 15 min with 10 nM epidermal growth factor (EGF) or a 5-fold excess of serum-free apoptotic DO11.10 cells (Apo). After washing, cell extracts were prepared from the adherent 3T3 cells. The presence of activated Akt was assessed by immunoblot analysis, probing with a phospho-Akt(Thr<sup>308</sup>)-specific rabbit antiserum. Apoptotic cells alone ("Apo only"; note this lane overrepresents the contribution of apoptotic cells in the "Apo" samples) also were probed and found to express no activated Akt. Equivalent loading of protein samples was confirmed by Ponceau S staining.

aspects of apoptotic recognition and response, as well as engulfment, occur independent of PSR expression.

### DISCUSSION

**PSR Is Dispensable for the Specific Anti-inflammatory Recognition and Clearance of Apoptotic Cells**—We evaluated the ability of non-professional phagocytes, as a function of their expression of PSR, to recognize, engulf, and respond to apoptotic cells. Studies of those processes in mice harboring targeted genetic disruptions of the PSR locus (13–15) have not been conclusive. In part, the perinatal lethality of homozygous PSR deficiency complicated those studies, prompting experiments in which particular populations of macrophages were generated selectively (13, 15).

Our approach was made possible by the appreciation that non-professional phagocytes are fully capable of non-inflammatory recognition and clearance of apoptotic cells.<sup>5</sup> We have described the specific functional recognition of apoptotic cells as a ubiquitous Toll-like receptor-independent innate immunity that discriminates live from effete cells without regard to self (2).<sup>5</sup> Furthermore, because PSR is broadly expressed in multiple tissues (15), an experimental focus on macrophages is needlessly limited.

We examined PSR contributions to an array of events associated with recognition, engulfment, and response to apoptotic cells, using a variety of stringent objective and quantitative criteria. Our experiments lead to the conclusion that PSR is involved neither in specific innate recognition nor engulfment of apoptotic cells.

The striking developmental pathologies associated with homozygous PSR deficiency, independent of strain background, strongly implicate PSR in processes of cellular differentiation and proliferation (13–15). In contrast, its link to cell death is more tenuous. While a genetic deficiency leading to a true defect in apoptotic phagocytosis or inflammatory modulation could be expected to manifest profound phenotypes (for examples, see Refs. 28 and 33), the reported anomalies in tissue clearance of dead cells in some PSR<sup>-/-</sup> animals (13, 14) are modest. Rather than being involved causally in developmental aberrations, we speculate that any persistent dead cells in PSR-deficient mice are consequent and transient manifestations of supernumerary developmental cell deaths and pathology. As a cautionary note, the predicted deleterious effects of a phagocytic defect likely would confer a strong selective

advantage for compensatory mechanisms, offering a further rationale against the use of selective cell populations in the study of genetic contributions.

Our data also suggest that PSR has no role in pro-inflammatory transcription. Notably, while Böse *et al.* (15) observed that stimulated secretion of inflammatory cytokines (TNF $\alpha$  and IL-6) from fetal liver macrophages was reduced modestly in the absence of PSR, they observed no differences on the level of cytokine gene transcription.<sup>6</sup>

**Reassessing the Role of PS in Apoptotic Cell Clearance**—We have argued that PS, which is taken to be the archetypal recognition molecule, is externalized on both apoptotic and necrotic cells and is not a specific ligand for the recognition of either one (1).<sup>5</sup> The appearance of determinants for recognition and inhibition of inflammation represents a post-translational gain-of-function, the revelation of cryptic activity during the process of physiological cell death specifically (1, 18).

The comparison of apoptotic and necrotic cells and their interactions with macrophages has been illuminating in this regard. Since externalization of PS occurs during apoptotic and necrotic cell death and PS vesicles are equally effective at preventing the uptake of both classes of targets, we concluded that PS displayed on the surface of both apoptotic and necrotic cells is not involved in the specific recognition of either one (1). Consistent with these findings, more recent data have demonstrated that PS, and tethering molecules that may bind externalized PS, are involved in engulfment, triggering the phagocytosis (macropinocytosis) of already bound targets (22, 28). In this light, the assumption that a PS-specific receptor may be linked to anti-inflammatory responsiveness may not be justified.

Furthermore, that recognition of, and response to, apoptotic cells occurs in the absence of serum (see Fig. 6) suggests that serum-derived tethering molecules are dispensable for the functional apoptotic recognition and that requisite specific ligand(s) for recognition and anti-inflammatory modulation are expressed endogenously by apoptotic cells.<sup>5</sup>

**Revisiting mAb 217 and the Concept of the PS-specific Receptor**—Our results exclude PSR from a functional role in innate apoptotic cell recognition and engulfment. Notable structural features of the PSR gene product include multiple nuclear localization signals (11) and a cupin metalloenzyme superfamily jmjC domain, predictive of a role in chromatin remodeling (30, 34). While no evidence exists for its involvement in inflammatory gene transcription or apoptotic modulation, a role for PSR in developmental regulation of gene expression is entirely consistent with the profound developmental defects associated with homozygous PSR deficiency. Apparent specific binding by PSR to PS has been reported previously, but the absence of biochemical characterization of that binding activity leaves its significance uncertain (27). In any case, we suggest that the designation of this gene product as a PSR is inappropriate.

It remains unclear whether this protein was identified incorrectly as the antigenic target of mAb 217 and whether mAb 217 recognizes an authentic engulfment receptor. As we have noted, the ability to ingest apoptotic cells is not restricted to professional phagocytes and extends even to non-phagocytic lymphocytes.<sup>5</sup> While a variety of cells express the mAb 217 antigen, it is not expressed on lymphoid cells (10). In particular, we note that human Jurkat T cells are fully competent to recognize and respond to apoptotic cells selectively,<sup>5</sup> although they do not express mAb 217 antigen (10). A second reservation pertains to the relevance of the observed TGF $\beta$  release stimulated by mAb 217. TGF $\beta$  release is not a proximal response to apoptotic recognition, and it is not involved in the establishment of apoptotic anti-inflammatory modula-

<sup>6</sup> A. Lengeling, personal communication.

tion (2). Thus, it is not a definitive criterion by which to implicate an apoptotic recognition molecule. Further study is needed to resolve these issues.

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**Molecular Basis of Cell and  
Developmental Biology:  
The Presumptive Phosphatidylserine  
Receptor Is Dispensable for Innate  
Anti-inflammatory Recognition and  
Clearance of Apoptotic Cells**

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