



# Exposure of phosphatidylserine is a general feature in the phagocytosis of apoptotic lymphocytes by macrophages

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Received 15.5.98; revised 8.7.98; accepted 11.11.98

Edited by B.A. Osborne

## Abstract

Although different macrophages exploit different cell surface receptors to recognize apoptotic lymphocytes, indirect evidence suggested that the phosphatidylserine (PS) that appears on the surface of lymphocytes undergoing apoptosis participates in specific recognition by all types of macrophages. To test this possibility directly, annexin V, a protein that specifically binds to PS, was used to mask this phospholipid on the apoptotic cell surface. Preincubation of apoptotic lymphocytes with annexin V blocked phagocytosis by elicited mouse peritoneal macrophages, macrophages of the mouse J774 cell line and mouse bone marrow macrophages. Similarly, annexin V was able to inhibit phagocytosis of lipid-symmetric erythrocytes, another target cell upon which PS is exposed. Together these results demonstrate directly that macrophages of all types depend on the PS exposed on the surface of apoptotic lymphocytes for recognition and phagocytosis.

**Keywords:** annexin V; programmed cell death; phagocytosis; phospholipids; membrane asymmetry; macrophage receptors

**Abbreviations:** PS, phosphatidylserine; PC, phosphatidylcholine; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMEM, Dulbecco's Minimal Essential Medium

## Introduction

In normal, healthy lymphocytes the aminophospholipid, phosphatidylserine (PS), is restricted to the inner leaflet of the plasma membrane by an ATPase, termed the aminophospholipid translocase, which specifically transports aminophospholipids from the outer to the inner leaflet of the bilayer.<sup>1–3</sup> This asymmetric distribution of PS can be abolished by activation of a non-specific lipid

flipsite, termed the scramblase. As part of the apoptotic program, the translocase is inactivated and the scramblase is activated,<sup>2</sup> bringing PS to the surface of apoptotic lymphocytes.<sup>4–6</sup>

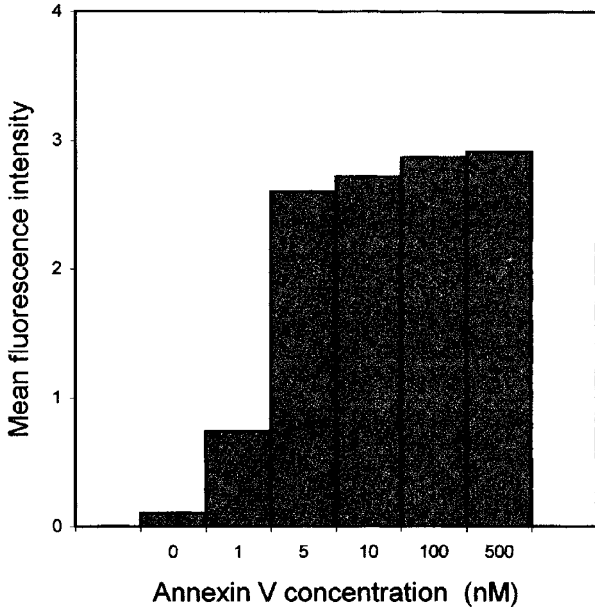
That exposed PS serves as a signal for phagocytosis was demonstrated by the ability of PS vesicles to specifically inhibit uptake of apoptotic lymphocytes by activated macrophages such as thioglycollate-elicited mouse peritoneal macrophages and activated mouse bone marrow macrophages.<sup>4,7–9</sup> On the other hand, uptake of apoptotic lymphocytes by unactivated macrophages such as human monocyte-derived macrophages, unactivated mouse bone marrow macrophages, and macrophages of the murine cell line J774 is not inhibited by PS vesicles,<sup>7,9</sup> despite the fact that these macrophages preferentially phagocytose PS vesicles.<sup>10–13</sup> These results suggested that only activated macrophages are able to recognize apoptotic lymphocytes by the PS exposed on their surface, even though unactivated macrophages are also able to specifically recognize PS. However, more recent studies provided an alternative explanation for this apparent discrepancy.

Unlike vesicles composed of PS, lipid-symmetric erythrocytes, on whose surface PS has been exposed by activation of the scramblase,<sup>14,15</sup> do inhibit phagocytosis of apoptotic thymocytes by unactivated (but not by activated) macrophages.<sup>9</sup> Since phagocytosis of lipid-symmetric erythrocytes by unactivated macrophages can be completely abolished by co-incubation with PS vesicles,<sup>13</sup> lipid-symmetric erythrocytes are recognized by the PS exposed on their surface. This result in turn suggests that unactivated macrophages also recognize apoptotic lymphocytes by the PS exposed on their surface, since their phagocytosis is inhibited by lipid-symmetric erythrocytes. Still, the evidence that unactivated macrophages recognize apoptotic lymphocytes by PS remains circumstantial in that it is difficult to rule out the possibility that the exposure of PS on lipid-symmetric erythrocytes induces or is accompanied by the alteration of some other surface component which is then responsible for their ability to inhibit phagocytosis of apoptotic lymphocytes. To demonstrate more directly that PS itself is recognized by unactivated macrophages, we used annexin V to mask the PS exposed on the surface of apoptotic lymphocytes and lipid-symmetric erythrocytes.

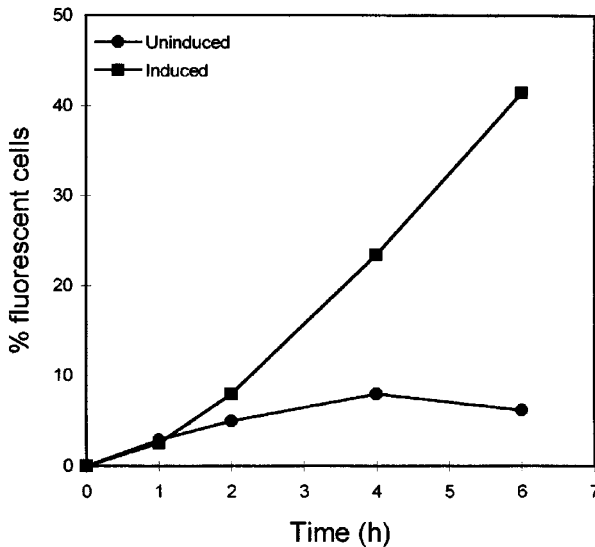
Annexin V is a member of a family of Ca<sup>2+</sup>-dependent, phospholipid-binding proteins. It specifically binds PS,<sup>16</sup> and its ability to bind to PS on the surface of lipid-symmetric erythrocytes and apoptotic lymphocytes has been amply documented.<sup>17,18</sup> Here we show that its binding to PS blocks recognition and phagocytosis of these cells by both activated and unactivated macrophages, demonstrating that in fact PS participates in the recognition of apoptotic lymphocytes by both types of macrophages.

### Results

The exposure of PS on the surface of lymphocytes undergoing apoptosis has been well documented,<sup>2,5-7,18-20</sup> including the primary thymocytes used here.<sup>21-23</sup> To establish the



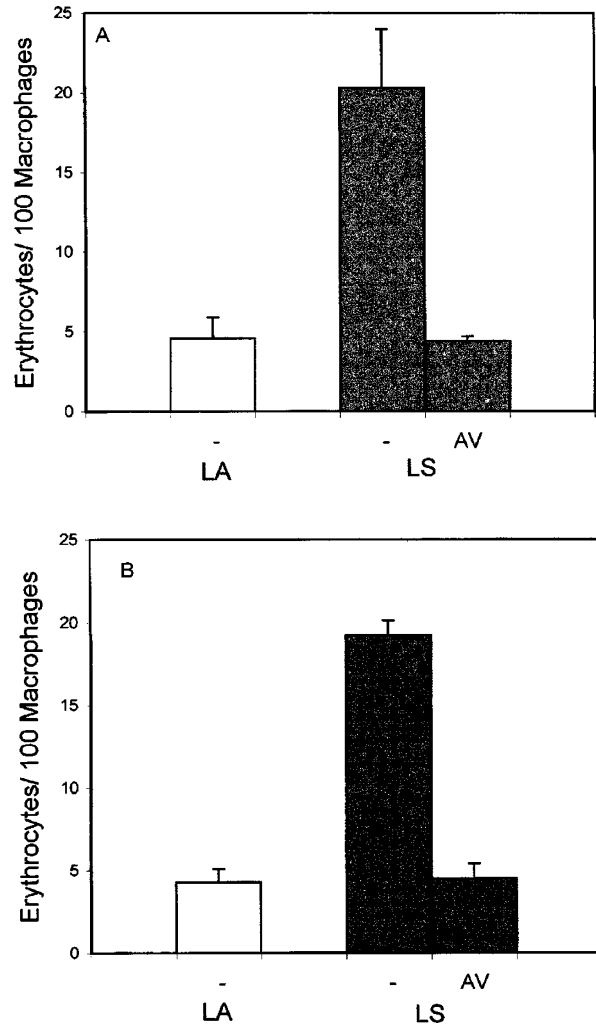
**Figure 1** Concentration dependence of annexin V binding to apoptotic thymocytes. Thymocytes, cultured for 6 h in the presence of  $10^{-6}$  M dexamethasone, were incubated with the indicated concentration of biotinylated annexin V, or in the absence of biotinylated annexin V, followed by staining with fluoresceinated streptavidin



**Figure 2** Binding of annexin V to thymocytes as a function of time of induction of apoptosis. Aliquots of thymocytes, incubated with  $10^{-6}$  M dexamethasone (■), were taken at various times and incubated in the presence of 100 nM biotinylated annexin V, followed by fluoresceinated streptavidin. Uninduced, control thymocytes (●), incubated in the absence of dexamethasone, are shown for comparison

concentration of annexin V optimal for binding to PS in this system, primary mouse thymocytes were incubated for 6 h with dexamethasone to induce apoptosis and then treated with biotinylated annexin V at various concentrations. The cells were then stained with fluoresceinated streptavidin, and examined by flow cytometry. As shown in Figure 1, fluorescence levels were nearly maximal after annexin V treatment at a concentration of 10 nM. To determine the kinetics of PS exposure on thymocytes induced to undergo apoptosis, saturating levels of annexin V were used to label thymocytes at various times after induction of apoptosis by dexamethasone. As shown in Figure 2, viable cells with PS exposed on their surface appeared as early as 2 h after addition of dexamethasone and increased in number thereafter for up to 6 h.

Since recognition of lipid-symmetric erythrocytes by both activated and unactivated macrophages can be blocked by PS vesicles,<sup>9,13</sup> binding of annexin V to these PS-exposing



**Figure 3** Phagocytosis of lipid-symmetric erythrocytes in the presence of annexin V. Lipid-symmetric (LS) erythrocytes were incubated in the presence or absence of 100 nM annexin V (AV), added to mouse peritoneal (A) or J774 (B) macrophages, and the number of erythrocytes phagocytosed in 30 min was counted. Lipid-asymmetric (LA) erythrocytes are shown for comparison

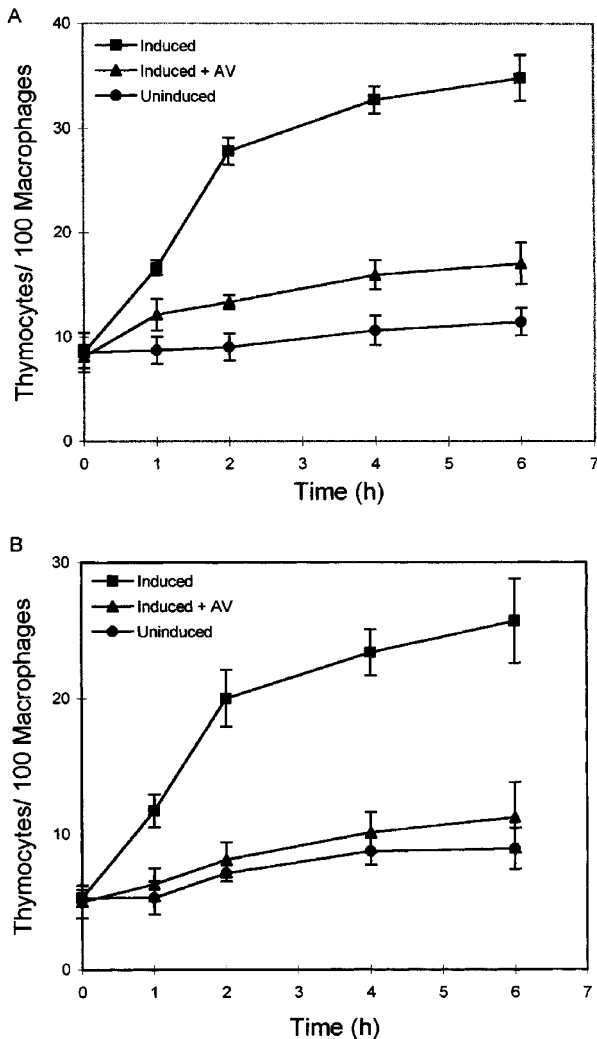
target cells should block uptake by both types of macrophages. This expectation was confirmed by the experiments shown in Figure 3, which demonstrate that recognition of lipid-symmetric erythrocytes by activated peritoneal macrophages (Figure 3A) and by unactivated J774 macrophages (Figure 3B) are equally sensitive to annexin V.

In contrast to the ability of PS vesicles to inhibit phagocytosis of lipid-symmetric erythrocytes by both unactivated and activated macrophages, PS vesicles inhibit phagocytosis of apoptotic thymocytes by activated macrophages only, while lipid-symmetric erythrocytes inhibit phagocytosis of apoptotic thymocytes by unactivated macrophages only.<sup>4,9</sup> To investigate the annexin V-sensitivity of these two recognition systems, thymocytes

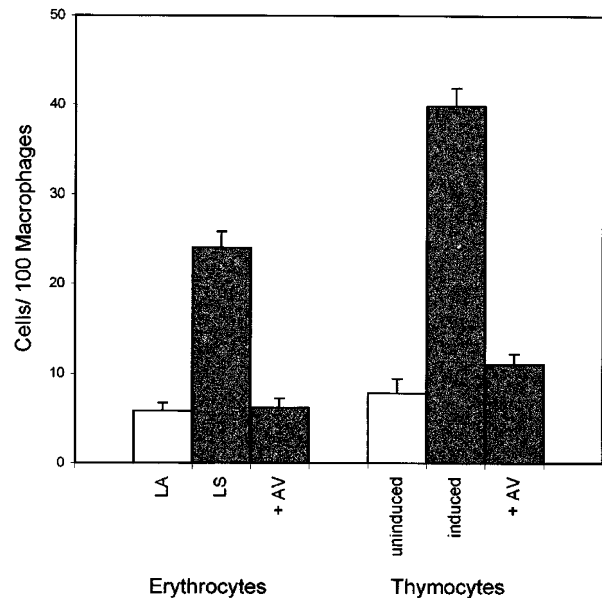
were treated with dexamethasone and at various times were presented to either activated peritoneal or unactivated J774 macrophages. As shown in Figure 4, PS vesicle-insensitive recognition by J774 cells is just as sensitive to annexin V (Figure 4B) as recognition by peritoneal macrophages (Figure 4A). Moreover, the appearance of annexin V-sensitive phagocytosis parallels closely the appearance of PS on the surface of apoptotic thymocytes (Figure 2).

Because the unactivated macrophages used in these experiments were a continuous cell line, it might be argued that their sensitivity to annexin V could be a property acquired during propagation. Therefore, phagocytosis by primary unactivated macrophages was examined. As shown in Figure 5, annexin V also blocked recognition of both lipid-symmetric erythrocytes and apoptotic thymocytes by primary mouse bone marrow macrophages. This result indicates that the sensitivity of recognition of apoptotic thymocytes to annexin V is common among macrophages which use a recognition system insensitive to PS vesicles, and thus demonstrates that sensitivity to annexin V is a general feature shared by all macrophages.

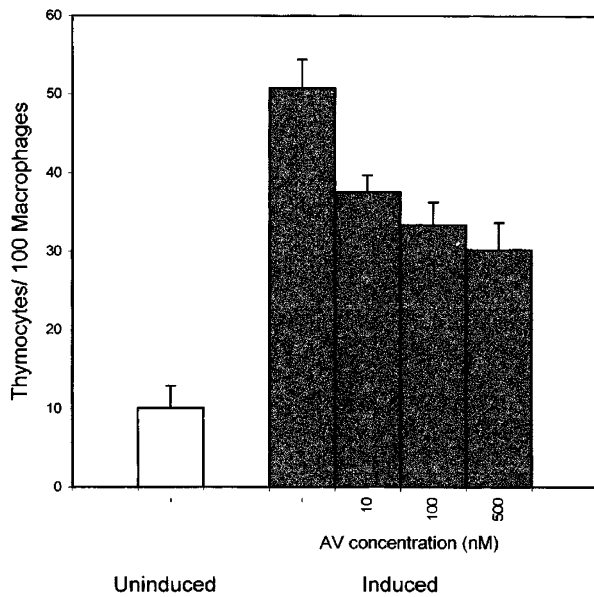
To ensure that the effects on recognition were the result of annexin V binding to PS and not, for example, the result of inhibition by some minor contaminant in the annexin V preparation, the concentration dependence of the inhibitory effects was examined. As shown in Figure 6, inhibition of recognition was largely complete at only 10 nM annexin V, with only small incremental effects at higher concentrations. Comparison with the concentration dependence for binding



**Figure 4** Phagocytosis of thymocytes in the presence of annexin V as a function of time of induction of apoptosis. Aliquots of thymocytes, incubated in the presence of  $10^{-6}$  M dexamethasone, were taken at various times, incubated in the presence (▲) or absence (■) of 100 nM annexin V (AV), and the number of thymocytes phagocytosed in 30 min by mouse peritoneal (A) or J774 (B) macrophages was counted. Uninduced control thymocytes (●), incubated in the absence of dexamethasone, are shown for comparison



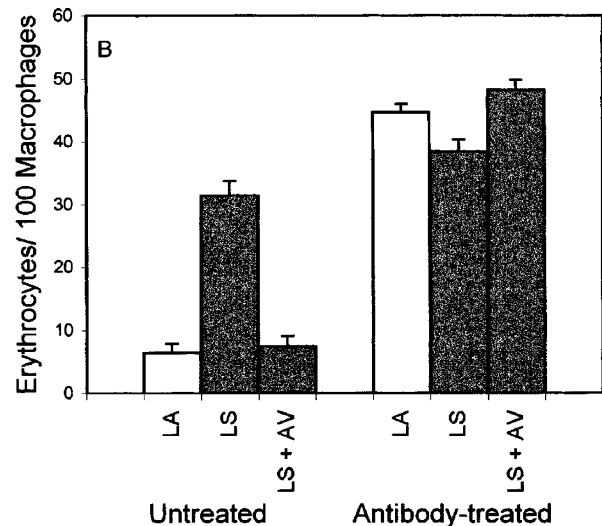
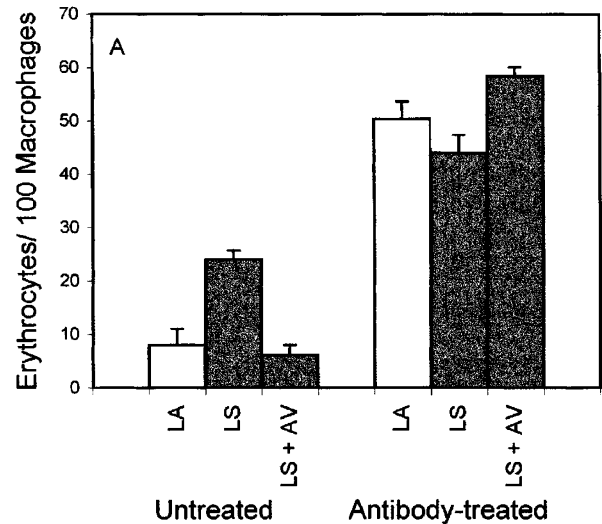
**Figure 5** Phagocytosis of lipid-symmetric erythrocytes and apoptotic thymocytes by mouse bone marrow macrophages in the presence of annexin V. Thymocytes, incubated with dexamethasone for 6 h, or lipid-symmetric (LS) erythrocytes, were incubated with or without 100 nM annexin V (AV), added to macrophages and the number of cells phagocytosed in 30 min was counted. Uninduced thymocytes, incubated in the absence of dexamethasone, and lipid-asymmetric (LA) erythrocytes are shown for comparison



**Figure 6** Phagocytosis of thymocytes by activated macrophages in the presence of various concentrations of annexin V. Thymocytes, cultured for 6 h in the presence of  $10^{-6}$  M dexamethasone, were incubated with the indicated concentrations of annexin V (AV), added to mouse peritoneal macrophages, and the number of thymocytes phagocytosed in 30 min was counted. Uninduced thymocytes, incubated in the absence of dexamethasone, are shown for comparison

of annexin V to the apoptotic cell surface (Figure 1) indicates that inhibition of recognition and binding to surface PS closely parallel one another. It should be noted, however, that the extent of inhibition is not as great as was seen in the experiment depicted in Figure 4, which reflects the variability from experiment to experiment seen with activated macrophages in this system.

A second possibility is that binding of annexin V to the cell surface is specific, but its inhibitory effects on phagocytosis are non-specific. One annexin V molecule can cover an area of membrane corresponding to as many as fifty phospholipid molecules<sup>16,24,25</sup> and, due to the formation of arrays, eight annexin V molecules can bind per PS residue.<sup>16,26</sup> Therefore, inhibition of phagocytosis by annexin V could be the result of steric hindrance to access of other recognition molecules or to non-specific inhibition of phagocytosis. To test this possibility, Fc receptor-mediated phagocytosis was measured in the presence of annexin V. Erythrocytes were pretreated with anti-glycophorin antibody and their phagocytosis by both activated and unactivated macrophages was measured. As shown in Figure 7, annexin V does not block Fc receptor-mediated phagocytosis by either activated or unactivated macrophages. In fact, Fc receptor-mediated phagocytosis was somewhat enhanced in the presence of annexin V. This result indicates that neither receptor-ligand interactions, nor phagocytosis itself, are blocked by annexin V binding to target cells, and thus suggests that the effects on recognition result from the ability of the annexin V molecule to mask PS exposed on the cell surface.



**Figure 7** Fc receptor-mediated phagocytosis of lipid-symmetric erythrocytes in the presence of annexin V. Lipid-symmetric (LS) erythrocytes were incubated in the presence or absence of anti-glycophorin A,B monoclonal antibody, then incubated in the presence or absence of 100 nM annexin V (AV), added to mouse peritoneal (A) or J774 (B) macrophages, and the number of erythrocytes phagocytosed in 30 min was counted. Lipid-asymmetric (LA) erythrocytes are shown for comparison

## Discussion

The studies presented here emphasize the importance and generality of the exposure of PS on the surface of apoptotic thymocytes as a fundamental signal for their recognition by all types of macrophages. This tenet has been generally conceded for activated macrophages, largely because recognition can be blocked by PS vesicles.<sup>4,9</sup> In contrast, the issue of whether unactivated macrophages also recognize PS exposed on apoptotic thymocytes has remained unresolved because recognition cannot be inhibited by PS vesicles.<sup>4,7,9</sup> However, the finding that masking PS on the target cells with annexin V is as

effective in blocking recognition by unactivated as activated macrophages implies that PS exposure on the surface during apoptosis is equally critical for both types of macrophages.

One unexpected feature of these studies is the effectiveness of inhibition by annexin V. Although recognition of lipid-symmetric erythrocytes by unactivated macrophages is completely sensitive to PS vesicles, such complete inhibition is rare – most inhibitors dramatically slow, but do not halt, the rate of phagocytosis of target cells. With the possible exception of recognition of apoptotic thymocytes by activated macrophages, the inhibition produced by annexin V is generally complete. This efficacy is all the more remarkable in view of the fact that coating of the same target cells with annexin V may even enhance phagocytosis when it is triggered by receptor-ligand interactions other than those employed in the removal of apoptotic cells.

This effectiveness of annexin V may derive from several sources. First, annexin V binds PS with high affinity, which may dramatically enhance its effectiveness in masking PS. Moreover, PS vesicles or lipid-symmetric erythrocytes do not operate by masking the PS signal on apoptotic cells at all, but rather compete with the signal for recognition at the macrophage surface. Annexin V may thus be more effective simply because it eliminates, rather than competes with that signal. However, that annexin V acts at the target cell, rather than the macrophage, surface may compound its effectiveness. Macrophage receptors occupied by inhibitors can be rapidly recycled following internalization, whereas membrane recycling in lymphocytes is much less prodigious. Moreover, annexin V bound to the lymphocyte surface does not appear to provoke internalization, nor lead to exposure of additional PS molecules.

Finally, the effectiveness of annexin V may derive from its interference at an early point in the recognition and phagocytosis process. Although this process is not yet well characterized, it must include at least initial receptor-ligand interaction, which in turn results in signaling to suppress inflammatory responses by the macrophage<sup>27</sup> as well as activation of the mechanisms involved in actual engulfment of the apoptotic cell. The many components required for completion of this process may be activated sequentially, such that blocking the earliest step in the pathway might be expected to have the greatest inhibitory effect. Thus, masking PS, preventing its recognition, might be expected to have particularly profound effects.

Several known macrophage surface proteins have been identified as participants in the recognition of apoptotic thymocytes; one of these is CD14.<sup>28</sup> The monoclonal antibody 61D3, which binds to CD14 and is specific for monocytes and macrophages, inhibits phagocytosis of apoptotic lymphocytes and lipid-symmetric erythrocytes by both activated and unactivated macrophages.<sup>9,29</sup> Macrophages on which CD14 is not expressed are completely unable to phagocytose either lipid-symmetric erythrocytes or apoptotic thymocytes (R.A. Schlegel, S. Krahlng, M.K. Callahan and P. Williamson, submitted). In this respect, eliminating CD14 is as effective as annexin V in blocking

recognition. This similarity could imply that CD14 itself recognizes PS, or that it acts at a correspondingly early step in the recognition process.

Although PS is clearly necessary for recognition of both lipid-symmetric erythrocytes and apoptotic thymocytes, PS appears to act in conjunction with other molecules on the apoptotic cell surface to create a complex recognition signal.<sup>9</sup> Moreover, it is clear that CD14 must interact with other, transmembrane proteins to transduce a signal, since it itself is not a transmembrane protein.<sup>30</sup> The nature of these cooperative interactions will provide fertile ground for future investigations.

## Materials and Methods

### Materials

Dexamethasone, propidium iodide, biotinamidocaproate N-hydroxy-succinimide ester, anti-glycophorin A,B (clone E3), bovine brain PS, egg phosphatidylcholine (PC) and conditioned medium from human giant cell tumor cells (product M7657) were purchased from Sigma Chemical Co. Diff-Quik staining reagents were purchased from Baxter. Streptavidin-FITC was purchased from Molecular Probes.

### Animals

Male CBA/J mice, 4–8 weeks of age, were maintained on food and water *ad libitum* in accordance with the guidelines of the Institutional Animal Care and Use Committee.

### Preparation of biotinylated annexin V

Annexin V was produced and purified as described by Burger *et al.*<sup>31</sup> Briefly, an overnight culture of *E. coli* TG1 containing a plasmid encoding human placental annexin V (clone pRK6; American Type Culture Collection) grown in LB medium containing 50 µg/ml of ampicillin was diluted fivefold into 1 l of fresh LB medium. After 3 h of growth, IPTG was added to a final concentration of 1 mM. The cells were harvested after 4 h of additional growth by centrifugation (5000 × *g*, 4°C, 5 min), and the pellet was resuspended in an equal volume of spheroplast buffer (0.5 mM EDTA, 750 mM sucrose, 200 mM Tris, pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml immediately prior to the addition of seven volumes of spheroplast buffer diluted 1:1 and incubated for 30 min on ice. Spheroplasts were harvested by centrifugation (14,000 × *g*, 4°C, 30 min) and the pellet resuspended in 10 ml of ultracentrifugation buffer (2 mM EDTA, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1 mg/ml of RNase, 0.1 mg/ml of DNAase I, 2 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml of pepstatin A, 0.1% (w/v) Triton X-100, 20 mM Tris, pH 8.0) for overnight centrifugation (100,000 × *g*, 4°C). Annexin V was purified from the supernatant based on its affinity for PS. Three mg of phospholipid (PS:PC; 2:1) stored in chloroform were dried down under N<sub>2</sub> and resuspended in 5 ml of phosphate-buffered saline (PBS; 7.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 10 mM KCl) by vortexing. Small unilamellar vesicles were prepared using a probe sonicator, added to the supernatant from overnight centrifugation and adjusted to 5 mM CaCl<sub>2</sub>. After 30 min on ice, the vesicles were harvested by centrifugation (40,000 × *g*, 4°C, 45 min) and washed once in PBS containing 5 mM CaCl<sub>2</sub>. The washed vesicle pellet was resuspended in extraction buffer (10 mM EDTA, 100 mM NaCl, 3 mM MgCl<sub>2</sub>, 20 mM Tris, pH 8.0) and centrifuged to remove the vesicles

(50,000 × g, 4°C, 60 min). The supernatant was dialyzed overnight in 75 mM phosphate buffer, pH 7.5; analysis by SDS-PAGE and Coomassie staining indicated the product to be >90% pure. To the supernatant was added 10 μl of a stock solution of biotinamidocaproate N-hydroxysuccinimide ester (20 mg/ml in anhydrous dimethylformamide) per mg of annexin V. Following incubation at room temperature for 60 min while shaking, free biotin was removed by dialysis against 75 mM phosphate buffer, pH 7.5.

### Isolation and induction of apoptosis in thymocytes

Thymuses were removed from 4–6-week-old mice, dissociated in PBS containing 5% fetal bovine serum (FBS); cells were collected by centrifugation, resuspended in 17 mM Tris, 140 mM NH<sub>4</sub>Cl, pH 7.2, to lyse erythrocytes, centrifuged, and resuspended at 10<sup>7</sup> cells/ml in Dulbecco's Minimal Essential Medium (DMEM) containing 10% FBS. Apoptosis was induced by addition of 10<sup>-6</sup> M dexamethasone and cells were incubated at 37°C in 5% CO<sub>2</sub>. To monitor apoptosis, 10<sup>6</sup> cells were washed with PBS and fixed by resuspending in equal volumes of PBS and 70% ethanol. Fixed cells were then centrifuged and resuspended in 200 μl of PBS, stained with propidium iodide at a final concentration of 18 μg/ml and analyzed by flow cytometry.

### Preparation of lipid-symmetric and lipid-asymmetric erythrocytes

Cells were prepared using erythrocytes from fresh human venous blood obtained from volunteers according to institutional guidelines. Blood was collected into ice-cold PBS containing 10 U/ml of heparin, centrifuged and the supernatant and the top 10% of erythrocytes were removed. An equal volume of PBS was added to the remaining pellet and an appropriate number of cells was removed, centrifuged and the supernatant removed. Four volumes of lysis buffer (0.1 × PBS containing 0.1 mM EGTA, 1 mM MgCl<sub>2</sub>, and 0.1% BSA, plus 1 mM CaCl<sub>2</sub> for lipid-symmetric erythrocytes only) were added to the pellet, and following vortexing, the lysing cells were placed on ice. After 2 min the cells were resealed by addition of 0.4 volumes (relative to the size of the original pellet) of 10 × PBS, the tube was inverted gently a few times and placed at 37°C. After 30 min 100 volumes of PBS were added, the cells pelleted, washed three times with PBS and then resuspended at a concentration of 10<sup>8</sup> cells/ml in PBS.

### Culture of mouse peritoneal, bone marrow, and J774A.1 macrophages

Inflammatory macrophages were elicited in the peritoneal cavity of 6–8-week-old mice by intraperitoneal injection of 1 ml of 3% Brewer's thioglycollate. Cells were harvested 5 days later by peritoneal lavage using 10 ml of ice-cold PBS containing 10 U/ml of heparin. The exudate was centrifuged at 4°C for 10 min, cells washed in RPMI 1640 and suspended in RPMI 1640 containing 5% FBS at a concentration of 4 × 10<sup>6</sup> cells/ml, then 150 μl (6 × 10<sup>5</sup> cells) pipetted onto 18-mm bicarbonate-treated glass coverslips kept in 60-mm petri dishes. After 2 h at 37°C, nonadherent cells were removed by aspiration, and the medium replaced with 150 μl/coverslip of fresh RPMI 1640 containing 5% FBS. Cultures were incubated overnight at 37°C in 5% CO<sub>2</sub> prior to use. Mouse bone marrow macrophages were harvested as described by Stewart *et al.*<sup>32</sup> and cultured for 5–7 days as described by Fadok *et al.*<sup>8</sup> except that conditioned medium from human giant cell tumor cells was used as a source of macrophage colony stimulating factor. Cells of the J774A.1 mouse monocyte-derived macrophage cell line (American Type Culture Collection) were grown in 10% FBS in DMEM at 37°C in 5% CO<sub>2</sub>. Twenty-four hours prior to phagocytosis

assays, triplicate coverslip cultures of 3 × 10<sup>5</sup> bone marrow or J774 macrophages were prepared.

### Phagocytosis assays

Thymocytes (10<sup>6</sup>) or erythrocytes (15 × 10<sup>6</sup>) were overlaid onto coverslip cultures of 3 × 10<sup>5</sup> macrophages. For inhibition studies, thymocytes or erythrocytes were incubated with various concentrations of annexin V in PBS supplemented with 2 mM CaCl<sub>2</sub> for 20 min at room temperature before being overlaid onto macrophages. To measure Fc receptor-mediated phagocytosis, erythrocytes were pretreated with a 1:400 dilution of a 0.5 mg/ml stock solution of anti-glycophorin monoclonal antibody for 15 min at room temperature, washed, and incubated with or without annexin V in PBS supplemented with 2 mM CaCl<sub>2</sub> for an additional 20 min at room temperature, then overlaid onto macrophages. After 30 min at 37°C in 5% CO<sub>2</sub>, cultures with thymocytes were washed three times in PBS, fixed in 1.8% formaldehyde and stained with Diff-Quik (Baxter). Cultures with erythrocytes were washed in 17 mM Tris, 140 mM NH<sub>4</sub>Cl, pH 7.2, to lyse non-phagocytosed erythrocytes, fixed in acidic methanol and stained with benzidine. Cells were counted as phagocytosed as previously described in detail.<sup>9</sup>

### Annexin V staining

10<sup>6</sup> thymocytes were incubated with the specified concentration of biotinylated annexin V for 15 min at room temperature in 100 μl of staining buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). After washing once, the cells were incubated with 1 μg of streptavidin-FITC in 100 μl of staining buffer for 15 min at room temperature and then brought to 500 μl. Propidium iodide was added at 10 μg/ml immediately prior to flow cytometric analysis in order to gate out dead cells.

### Flow cytometry

A minimum of 10 000 cells/sample were analyzed using an EPICS XL-MCL flow-cytometer (Coulter Electronics, Hialeah, FL, USA) fitted with a single 15 mW argon ion laser providing excitation at 488 nm. A 620 nm bandpass filter was used to analyze DNA staining of fixed thymocytes with propidium iodide. FITC staining was monitored through a 525 nm bandpass filter. Mean fluorescence intensity of cells stained with biotinylated-annexin and streptavidin-FITC, in arbitrary units relative to base level fluorescence of cells stained with streptavidin-FITC alone, was determined from a plot of fluorescence intensity *versus* cell number using the XL2 software package provided with the EPICS XL-MCL.

### Acknowledgements

Supported by the United States National Science Foundation. We thank Elaine Kunze for help with flow cytometry and Heather Conrad for technical assistance.

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