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CD14 is a component of multiple recognition systems used by macrophages to phagocytose apoptotic lymphocytes

Robert A. Schlegel*[,1], Stephen Krahling[1], Melissa K. Callahan[1] and Patrick Williamson[2]

[1] Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania 16802, USA
[2] Department of Biology, Amherst College, Amherst, Massachusetts 01002, USA
* corresponding author: R. A. Schlegel, Department of Biochemistry and Molecular Biology, 428 South Frear Laboratory, Penn State University, University Park, Pennsylvania 16802 USA; tel: 814 865-6974; fax: 814 863-7024; e-mail: ur3@psu.edu

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Abstract

Expression of the aminophospholipid phosphatidylserine (PS) on the surface of apoptotic lymphocytes and lipid-symmetric erythrocytes triggers their phagocytosis by macrophages. Phagocytosis by both activated and unactivated macrophages, which utilize different recognition systems, can be blocked by certain monoclonal antibodies directed against the LPS receptor, CD14. Here we investigate the requirement for CD14 in the phagocytosis of both apoptotic thymocytes and lipid-symmetric erythrocytes by both activated and unactivated macrophages. We show that phagocytosis of lipid-symmetric erythrocytes by both activated and unactivated macrophages is completely abolished when CD14 is removed from macrophages by cleaving its glycosylphosphatidylinositol tether with phospholipase C. This treatment also substantially reduces phagocytosis of apoptotic lymphocytes by both types of macrophages. Unactivated LR-9 mouse macrophages which are deficient in CD14 expression are completely unable to phagocytose either apoptotic thymocytes or lipid-symmetric erythrocytes. These results argue that CD14 is an absolute requirement for the phagocytosis of lipid-symmetric erythrocytes by both activated and unactivated macrophages, despite their different recognition systems, that CD14 contributes at least substantially to the phagocytosis of apoptotic lymphocytes by both activated and unactivated macrophages, and that activated macrophages may also possess an alternate, CD14-independent mechanism for phagocytosis of apoptotic lymphocytes.

Keywords: monocytes/macrophages; T lymphocytes; apoptosis; phagocytosis

Abbreviations: mAb, monoclonal antibody; PS, phosphatidylserine; GlcNAc, N-acetylglucosamine; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C

Introduction

The mechanisms by which macrophages recognize and phagocytose apoptotic lymphocytes are complex. Studies of an array of reagents which inhibit the process indicate that macrophages use at least two different recognition systems, each inhibited by a different set of reagents.[1–4] One system is used by 'unactivated' macrophages, including human monocyte-derived macrophages, mouse bone marrow macrophages and cells of the J774 mouse macrophage cell line. The tetrapeptide RGDS, as well as cationic sugars and amino acids, inhibit uptake of apoptotic lymphocytes by this system. Anti-vitronectin receptor antibody has the same effect, implicating this integrin in recognition by unactivated macrophages. The other system is used by 'activated' macrophages, including elicited mouse peritoneal macrophages and activated bone marrow macrophages. The sugar N-acetylglucosamine (GlcNAc) inhibits uptake by this system, implicating recognition of a carbohydrate on apoptotic lymphocytes by a lectin-like receptor on activated macrophages. Although the two recognition systems are clearly different, the aminophospholipid phosphatidylserine (PS) participates in both.

In those eukaryotic cells thus far examined, including lymphocytes, PS is restricted to the inner leaflet of the plasma membrane[5] by a P-type ATPase which specifically transports PS from the outer to the inner leaflet of the bilayer.[6] During apoptosis in lymphocytes, this aminophospholipid translocase is inactivated and a non-specific lipid flipsite, called the scramblase, is activated, bringing PS to the cell surface.[7] The importance of PS on the apoptotic cell surface for recognition by macrophages was first revealed by the ability of PS vesicles to inhibit the phagocytosis of apoptotic lymphocytes by activated macrophages.[1–4] Subsequent studies showed that erythrocytes with PS on their surface (lipid-symmetric erythrocytes) could inhibit phagocytosis of apoptotic lymphocytes by unactivated macrophages.[4] Each of these two PS-presenting inhibitors is effective against only one or the other type of macrophage, emphasizing the distinctive nature of the recognition systems on unactivated *vs* activated macrophages. However, the importance of PS in both systems was recently demonstrated directly by the finding that annexin V, which binds specifically to PS, blocks the phagocytosis of apoptotic lymphocytes by both activated and unactivated macrophages.[8]

In these mutually exclusive recognition systems, one reagent is notable for its ability to inhibit uptake of apoptotic lymphocytes by both unactivated and activated macrophages. The 61D3 monoclonal antibody (mAb), generated against human monocytes,[9] was first demonstrated to inhibit the uptake of apoptotic lymphocytes by unactivated human monocyte-derived macrophages.[10] Subsequently, the antibody was shown to inhibit the uptake of apoptotic lymphocytes by both unactivated J774 mouse macro-

phages and activated mouse peritoneal macrophages,[4] showing at once that the epitope recognized was not species-specific and that the antibody was effective against both the activated and unactivated recognition systems. In addition, the antibody was able to block phagocytosis of lipid-symmetric erythrocytes by both unactivated and activated macrophages.[4]

Recently, the molecule containing the epitope recognized by the 61D3 mAb was identified by expression cloning as CD14,[11] a macrophage surface receptor which binds LPS complexed with an LPS-binding serum protein.[12] In this same study, COS cells transfected with CD14 were shown to bind and phagocytose apoptotic lymphoma cells with greater efficiency than COS cells transfected with control vector.[11] The authors pointed out 'that CD14 does not appear to function as efficiently in the COS cell as it does in the macrophage (despite overexpression of the molecule on COS cells as compared with macrophages) indicates that additional macrophage molecules may cooperate with CD14 to effect the binding and phagocytosis of apoptotic cells.' We therefore sought to assess the importance of the CD14 molecule in the context of the complex collection of recognition molecules presented on the macrophage surface.

Inhibition of phagocytosis by 61D3 mAb is variable and only partial, handicapping evaluation of the contribution of CD14 to the phagocytosis of apoptotic cells by macrophages. Therefore, to directly evaluate the importance of CD14 to the several recognition systems deployed on the macrophage surface, we have asked (1) the extent to which the absence of CD14 from the macrophage cell surface affects phagocytosis of apoptotic cells, (2) whether unactivated and activated macrophages are affected to the same extent, and (3) whether phagocytosis of PS-presenting, lipid-symmetric erythrocytes is similarly affected.

Results

The recognition of apoptotic thymocytes by macrophages proceeds comparably with either of the two (mutually exclusive) recognition systems. Which system is in use can be readily determined by applying inhibitors, as shown in Figure 1 for phagocytosis by primary murine bone marrow macrophages. In Figure 1A, unactivated bone marrow macrophages recognize apoptotic thymocytes using the integrin-based system, as evidenced by the sensitivity of phagocytosis to inhibition by RGDS, but not by PS vesicles. As shown in Figure 1B, these same macrophages, following activation by β-1,3-glucan, switch to the second recognition system, as revealed by the disappearance of sensitivity to RGDS accompanied by the acquisition of sensitivity to inhibition by PS vesicles.[3]

While the same macrophages use different systems to recognize apoptotic thymocytes depending on their physiologic state, phagocytosis in both cases is dependent on the target of the 61D3 mAb, as shown by the ability of the antibody to inhibit phagocytosis by both unactivated (Figure 1A) and activated (Figure 1B) mouse bone marrow macrophages. The ability to block recognition of apoptotic cells by human macrophages is not a general property of other anti-human CD14 antibodies;[11] therefore, as ex-

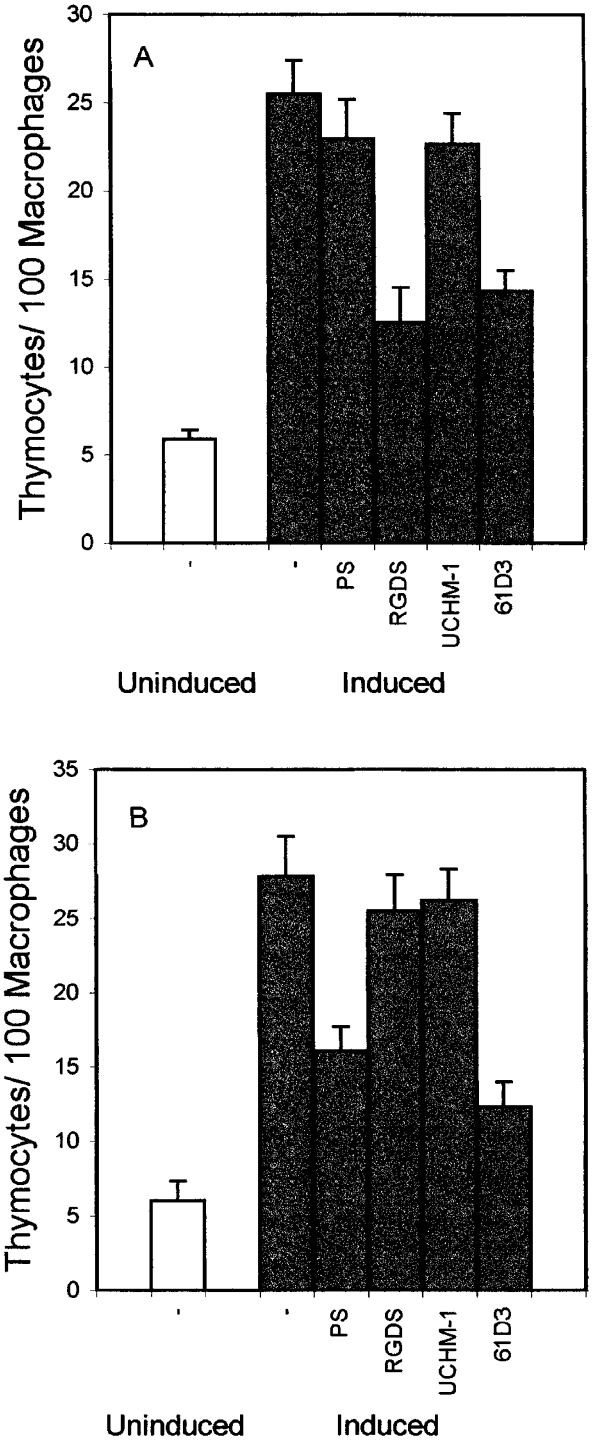

Figure 1 Phagocytosis of thymocytes by unactivated and activated mouse bone marrow macrophages in the presence of various inhibitors. Thymocytes, incubated in the presence (induced) or absence (uninduced) of 10^{-6} M dexamethasone for 6 h were mixed with PS vesicles or suspended in RGDS, 61D3 mAb antibody or UCHM-1 anti-CD14 mAb antibody; added to monolayer cultures of unactivated mouse bone marrow macrophages (**A**) or mouse bone marrow macrophages activated by treatment with beta-1,3-glucan as described in Materials and Methods (**B**)

pected, phagocytosis by both unactivated and activated mouse bone marrow macrophages is unaffected by the presence of UCHM-1, another anti-human CD14 mAb (Figure 1).

Although exposure of PS is the only change known to occur on the surface of apoptotic lymphocytes, the receptor recognized by 61D3 mAb could be sensing some other, as yet unrecognized, change on the apoptotic cell surface. A simpler PS-presenting target cell is provided by lipid-symmetric erythrocytes; phagocytosis of these cells by unactivated macrophages is completely blocked by PS vesicles,[13] implying that recognition in this system depends entirely on PS. Inhibition of phagocytosis of apoptotic lymphocytes by the 61D3 mAb was originally observed using human monocyte-derived macrophages,[10] and these unactivated macrophages are also known to preferentially phagocytose lipid-symmetric erythrocytes.[14] As shown in Figure 2, phagocytosis of lipid-symmetric erythrocytes by human monocyte-derived macrophages depends on recognition of PS, as evidenced by the sensitivity of the uptake to PS vesicles. Recognition in this model system is also blocked by the 61D3 mAb (Figure 2), implying that these macrophages use the same receptor to recognize both apoptotic thymocytes and lipid-symmetric erythrocytes.

The participation of the 61D3 macrophage antigen in the recognition of lipid-symmetric erythrocytes can also be observed in the murine bone marrow macrophage system. As shown in Figure 3, both the unactivated and activated forms of these primary cells will phagocytose lipid-symmetric erythrocytes in preference to control erythrocytes in which the lipid distribution is normal and PS does not appear on the surface. As in the case of apoptotic cells,

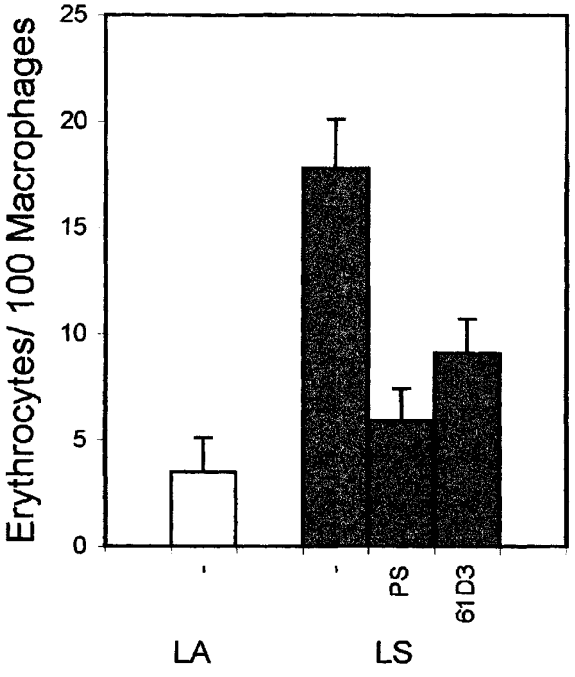

Figure 2 Phagocytosis of erythrocytes by human monocyte-derived macrophages in the presence of inhibitors. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes were mixed with PS vesicles or suspended in 61D3 mAb, added to monolayer cultures of human monocyte-derived macrophages

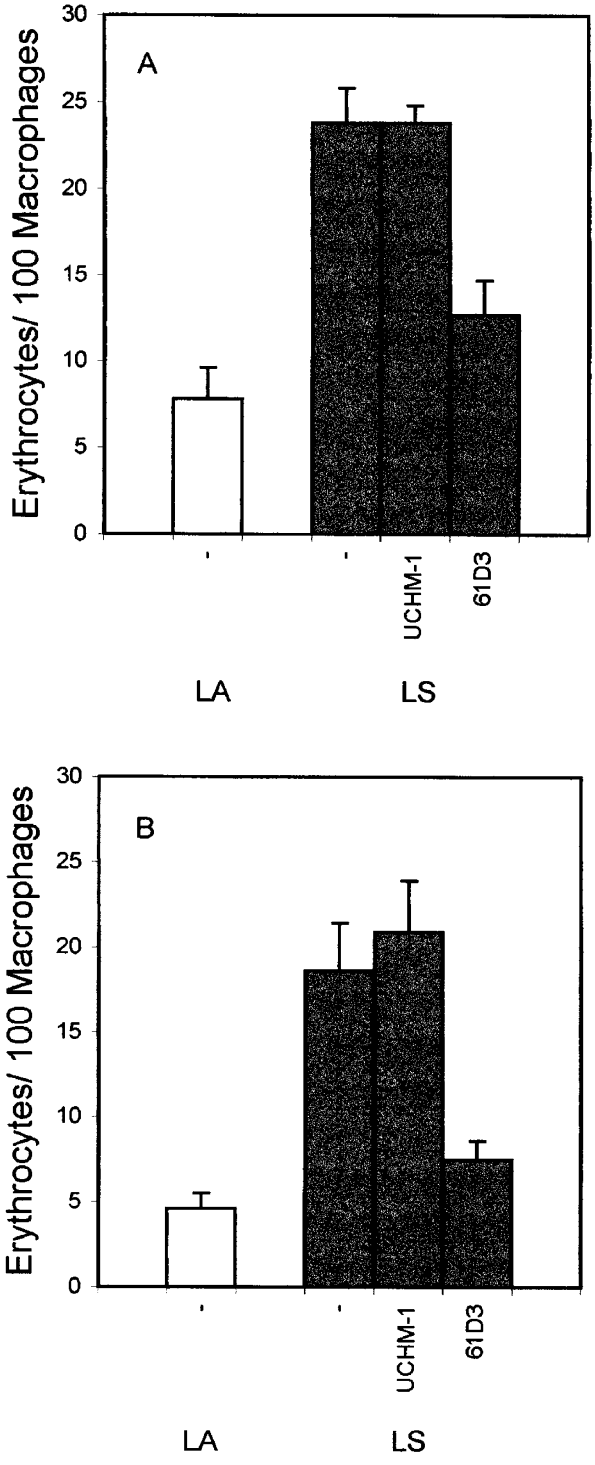

Figure 3 Phagocytosis of erythrocytes by unactivated and activated mouse bone marrow macrophages in the presence of monoclonal antibodies. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes were incubated with 61D3 mAb or UCHM-1 anti-CD14 mAb; added to monolayer cultures of unactivated mouse bone marrow macrophages (**A**) or mouse bone marrow macrophages activated by treatment with beta-1,3-glucan as described in Materials and Methods (**B**)

preferential uptake by both macrophage populations is sensitive to inhibition by 61D3 mAb. Moreover, this property of the 61D3 mAb is again not shared by another anti-human CD14 mAb: addition of the UCHM-1 mAb to human CD14 has no effect on recognition of these PS-presenting cells by either system.

One approach to assessing the role of CD14 in the phagocytosis of apoptotic lymphocytes and lipid-symmetric erythrocytes by macrophages is to take advantage of the fact that CD14 is not a transmembrane protein, but rather is tethered to the membrane by a glycosylphosphatidylinositol (GPI) linkage; treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) can therefore release CD14 from the cell surface,[12,15] permitting a direct evaluation of its importance to macrophage phagocytosis. As demonstrated previously[13] and shown in Figure 4A, phagocytosis of lipid-symmetric erythrocytes by unactivated J774 macrophages is entirely dependent on the PS exposed on the surface of these cells, since it is completely inhibitable by PS vesicles. In contrast, recognition of lipid-symmetric erythrocytes by these macrophages is unaffected by RGDS or GlcNAc.[4] As also shown in Figure 4A, treatment of J774 cells with PI-PLC to release CD14 completely abrogates the ability of these macrophages to preferentially phagocytose lipid-symmetric erythrocytes. To ensure that PI-PLC treatment did not compromise the general ability of cells to phagocytose, the phagocytosis of latex spheres by PI-PLC-treated J774 macrophages and untreated control cells was measured; no difference in the phagocytic index was seen between treated and untreated cells (data not shown).

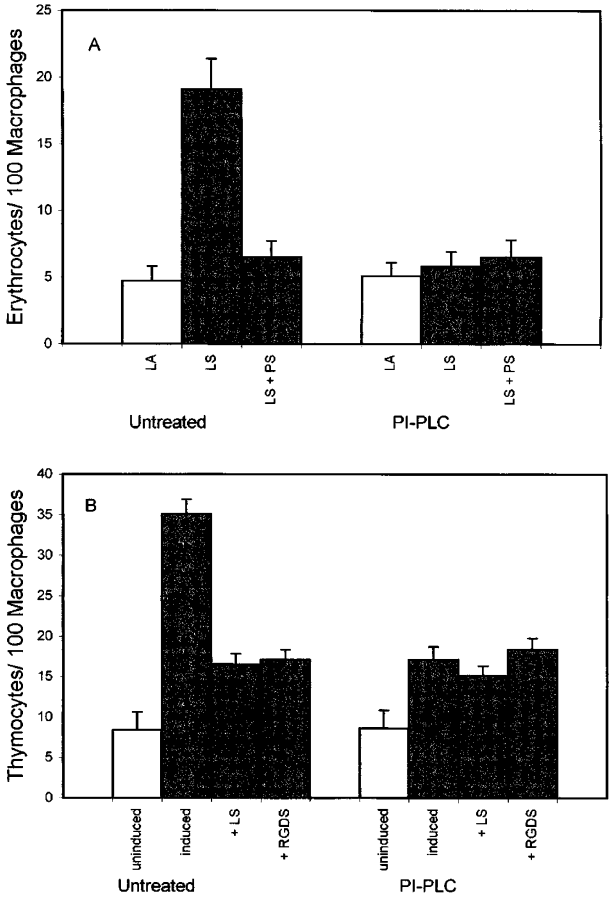

Figure 4 Phagocytosis of erythrocytes and thymocytes by J774 macrophages before and after treatment with PI-specific phospholipase C. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes (**A**) or induced or uninduced thymocytes (**B**) were mixed with PS vesicles or lipid-symmetric erythrocytes, or suspended in RGDS; added to monolayer cultures of J774 macrophages, either untreated or treated with PI-specific phospholipase C (PI-PLC) as described in Materials and Methods

Recognition of apoptotic thymocytes by unactivated J774 macrophages is more complex than recognition of lipid-symmetric erythrocytes in that phagocytosis is inhibited by RGDS or PS presented on lipid-symmetric erythrocytes[4] (Figure 4B). After treatment with PI-PLC to release CD14, the ability of these cells to recognize and phagocytose apoptotic thymocytes was substantially reduced, although not to the same extent as seen for recognition of lipid-symmetric erythrocytes (Figure 4A). The incomplete inhibition does not appear to be due to residual integrin or PS-mediated signaling, since addition of either RGDS or lipid-symmetric erythrocytes (or PS vesicles; not shown) did not reduce the phagocytosis by PI-PLC-treated macrophages further (Figure 4B).

The profile of inhibitors to which J774 macrophages are sensitive is characteristic of unactivated macrophages.[2,4] However, to be sure that the consequences of PI-PLC treatment could not be attributed to the fact that J774 macrophages are a cultured line of murine cells, the same experiments were repeated with human monocyte-derived macrophages. As shown in Figure 5A, PI-PLC treatment of these primary unactivated macrophages had the same dramatic effect on (PS vesicle-sensitive) recognition of lipid-symmetric erythrocytes as was observed with the J774 cell line. Similarly, PI-PLC treatment substantially reduced, but did not entirely eliminate, recognition of apoptotic thymocytes (Figure 5B), implying that these properties are general to the recognition system used by unactivated cells.

As 61D3 antibody is effective against both unactivated and activated macrophages, removal of CD14 from activated macrophages should similarly affect their recognition of both lipid-symmetric erythrocytes and apoptotic lymphocytes. The recognition of lipid-symmetric erythrocytes by activated macrophages is more complicated than their recognition by unactivated macrophages in that inhibition by PS vesicles is not complete and phagocytosis can be inhibited by GlcNAc as well[4] (Figure 6A). However, this additional complexity does not affect the outcome of CD14 removal; as in the case of unactivated macrophages, phagocytosis of lipid-symmetric erythrocytes by activated macrophages was completely abolished following PI-PLC treatment. As with unactivated macrophages, recognition of apoptotic thymocytes is also strongly, but not completely, inhibited by PI-PLC treatment (Figure 6B). In this case, residual phagocytosis is reduced somewhat further by PS vesicles or GlcNAc, indicating that a small fraction of the remaining activity is attributable to residual PS- and GlcNAc-sensitive signaling which is normally used by these cells.

Biochemical removal of CD14 by PI-PLC treatment has the disadvantage that it removes other GPI-linked proteins from the cell surface as well. Thus, the possibility that the removal of other GPI-linked proteins besides CD14 might contribute significantly to the effects seen cannot be ruled out. To test more specifically whether these effects can be entirely explained by the removal of CD14, advantage was taken of the LR-9 strain of J774 cells, which was selected for its resistance to LPS[16] and found to be deficient in the expression of CD14.[17] As shown in Figure 7, LR-9 cells are deficient in the surface expression of CD14 in comparison with the parental J774 cells. If the effects of PI-PLC treatment are a result of the removal of CD14, recognition of lipid-symmetric erythrocytes and apoptotic thymocytes by LR-9 cells should mimic that of PI-PLC-treated J774 cells. This prediction is confirmed by the results shown in Figure 8. The number of lipid-symmetric erythrocytes phagocytosed by LR-9 cells was no greater than the number of lipid-asymmetric control cells phagocytosed (Figure 8A). In fact, the number of lipid-symmetric and lipid-asymmetric control cells phagocytosed was lower than the number of lipid-asymmetric control cells phagocytosed by parental J774 cells. In the case of apoptotic thymocytes, as shown in Figure 8B, LR-9 macrophages are no better able to phagocytosis apoptotic than normal thymocytes, and take up both at a level lower than the parental J774 cells take up the same cells.

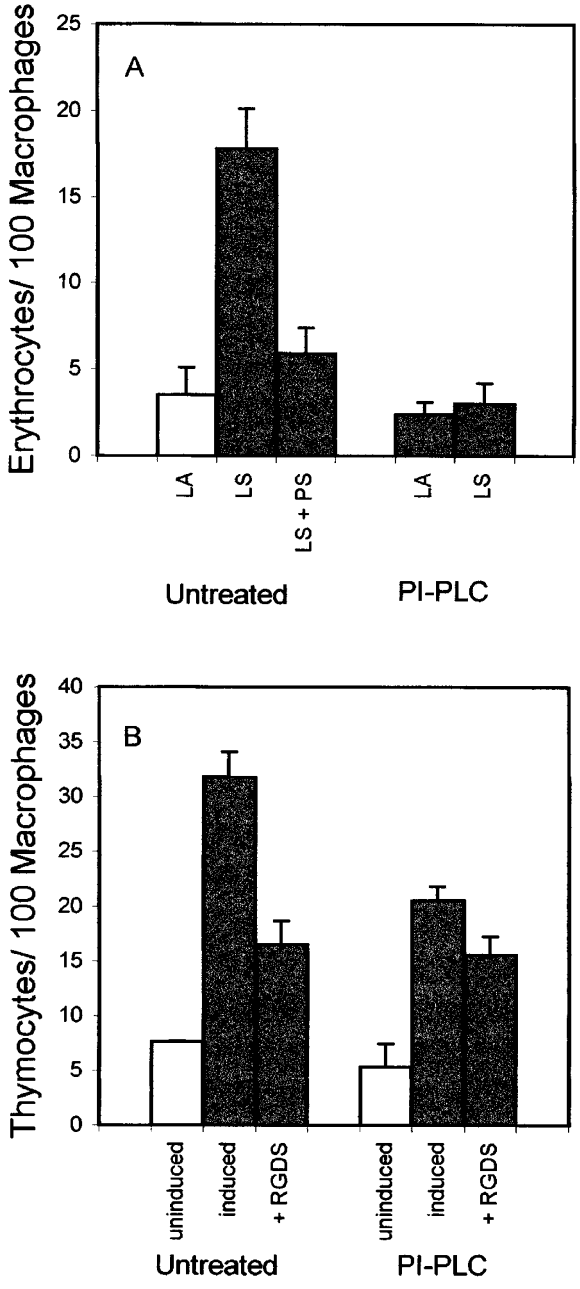

Figure 5 Phagocytosis of erythrocytes and thymocytes by human monocyte-derived macrophages before and after treatment with PI-specific phospholipase C. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes (**A**) or induced or uninduced thymocytes (**B**) were mixed with PS vesicles or suspended in RGDS; added to monolayer cultures of human monocyte-derived macrophages, either untreated or treated with PI-specific phospholipase C (PI-PLC) as described in Materials and Methods

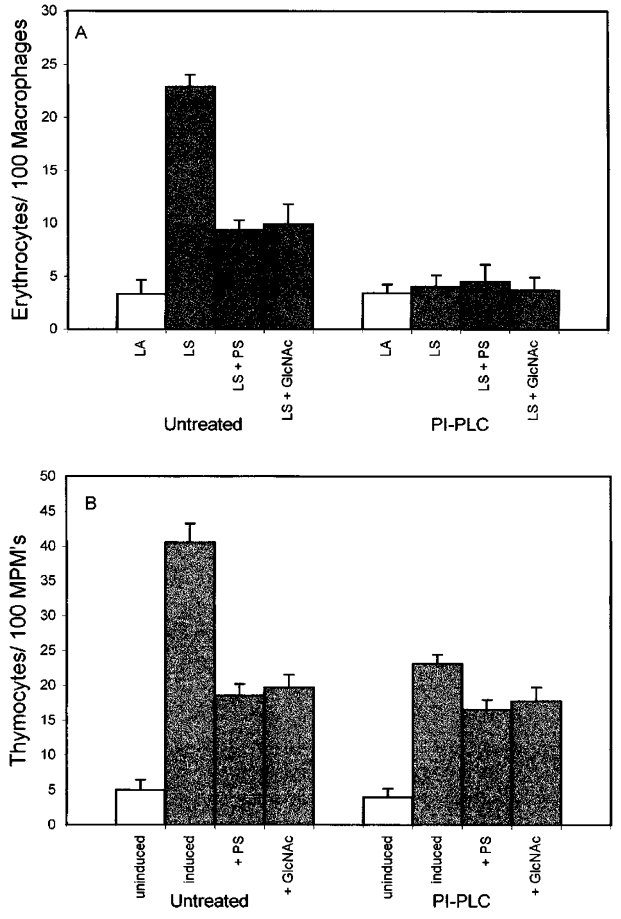

Figure 6 Phagocytosis of erythrocytes and thymocytes by elicited mouse peritoneal macrophages before and after treatment with PI-specific phospholipase C. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes (**A**) or induced or uninduced thymocytes (**B**) were mixed with PS vesicles or suspended in RGDS or GlcNAc; added to monolayer cultures of mouse peritoneal macrophages, either untreated or treated with PI-specific phospholipase C (PI-PLC) as described in Materials and Methods

To rule out the possibility that LR-9 cells are generally defective in phagocytosis, a comparison was made of the ability of the parental J774 and mutant LR-9 cells to take up erythrocytes by Fc receptor-mediated phagocytosis. As shown in Figure 9, erythrocytes coated with anti-glycophorin antibody were taken up just as effectively by LR-9 cells as parental J774 cells, indicating that the mutant cells are still competent phagocytes. The Fc receptor-mediated nature of this uptake was confirmed by pre-treating the macrophages with IgG to block Fc receptors. Phagocytosis of the opsonized erythrocytes was inhibited as expected.

Discussion

An unexpected feature of the recognition of apoptotic lymphocytes by macrophages is the diversity of the recognition signals and receptors involved, as implied by the diversity of inhibitors which block phagocytosis. As shown previously and confirmed here for a variety of mammalian macrophages, this diversity can be resolved into two distinct recognition systems, only one of which is utilized by a population of macrophages at any time. These distinct systems are united by their sensitivity to the mAb 61D3. The discovery that expression of CD14 on COS cells is necessary and sufficient for anti-61D3 reactivity has prompted the specific tests performed here to assess the importance of CD14 to both activated and unactivated macrophages in the phagocytosis of apoptotic lymphocytes as well as erythrocytes with PS exposed on their surface. The data presented here show that CD14 is an important participant in the several mechanisms used by macrophages for recognition of both apoptotic cells and model lipid-symmetric erythrocytes.

The simplest recognition system examined here is the recognition of lipid-symmetric erythrocytes by unactivated macrophages, where phagocytosis can be completely abolished by PS vesicles, but is completely insensitive to inhibitors of integrins or to carbohydrates. As shown here, phagocytosis of lipid-symmetric erythrocytes is also completely abolished by treatment of unactivated macro-

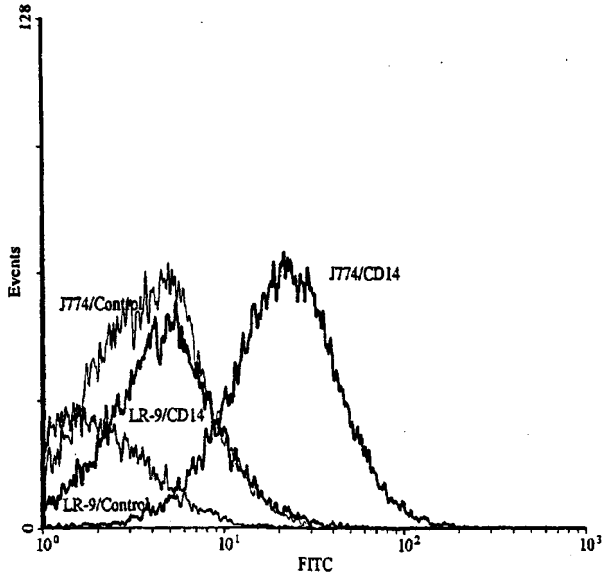

Figure 7 Expression of CD14 on J774 and LR-9 macrophages. J774 or LR-9 macrophages were stained with FITC-conjugated anti-CD14 mAb or FITC-conjugated isotype control mAb and analyzed for fluorescence by flow cytometry

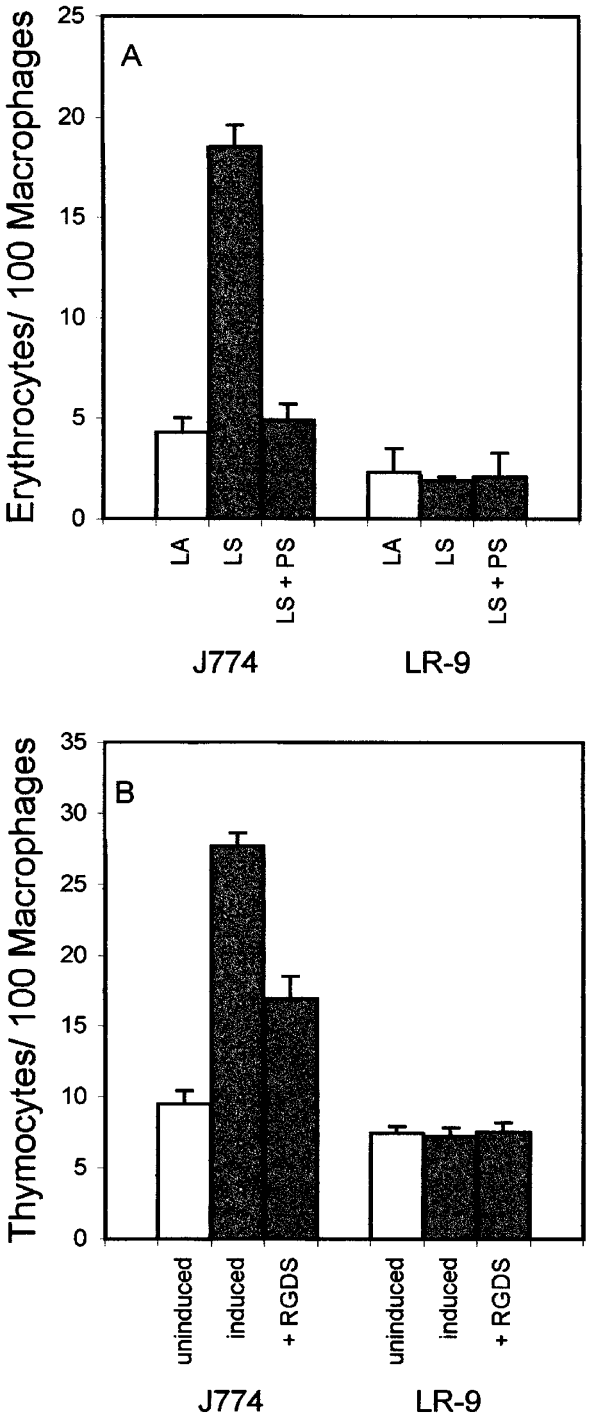

Figure 8 Phagocytosis of erythrocytes and thymocytes by J774 and LR-9 macrophages. Lipid-symmetric (LS) or lipid-asymmetric (LA) erythrocytes (**A**) or induced or uninduced thymocytes (**B**) were mixed with PS vesicles or suspended in RGDS; added to monolayer cultures of either J774 or LR-9 macrophages

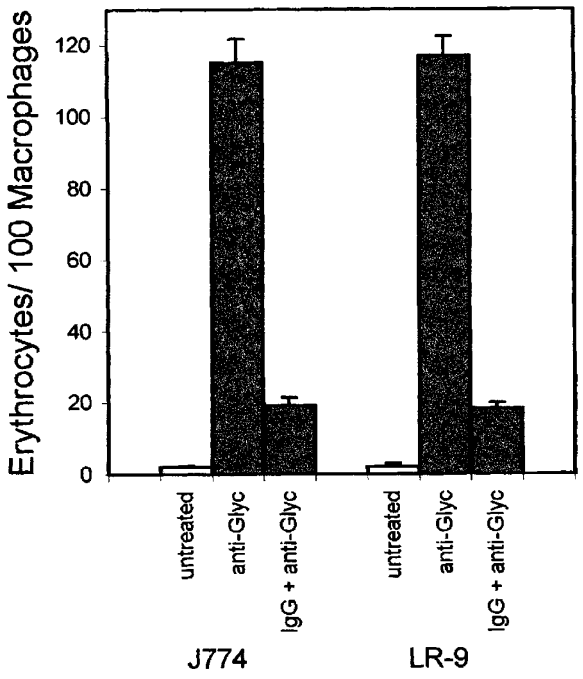

Figure 9 Fc receptor-mediated phagocytosis of erythrocytes by J774 and LR-9 macrophages. Untreated erythrocytes or erythrocytes treated with anti-glycophorin mAb were added to monolayer cultures of untreated macrophages or macrophages treated with IgG

phages with PI-PLC and is absent in LR-9 macrophages, demonstrating that the presence of CD14 on the macrophage surface is an absolute requirement for recognition of lipid-symmetric erythrocytes by the unactivated macrophage recognition system. Together, these results imply that both PS on the apoptotic cell surface and CD14 on the macrophage surface are required for recognition and phagocytosis. However, in neither case do the results speak to the question of whether either molecule of the signal/receptor complement is sufficient, because both could combine with other molecules on the respective cell surface to form a complex signal and a corresponding receptor complex.

These same unactivated macrophages apparently utilize a more complex signal/receptor complement for recognition of apoptotic lymphocytes in that RGDS becomes inhibitory and these same PS-presenting lipid-symmetric erythrocytes, but not PS-presenting vesicles, can inhibit phagocytosis of apoptotic thymocytes. The inhibitory effects of RGDS and lipid-symmetric erythrocytes are not additive,[4] suggesting they act on the same signal/receptor complement. That concept is sustained here in that neither lipid-symmetric erythrocytes nor RGDS reduced the phagocytosis of apoptotic thymocytes beyond that resulting from PI-PLC treatment. These findings suggest that CD14 on unactivated macrophages is a component of a single receptor complex for recognition of apoptotic thymocytes whose efficiency is similarly impaired by two quite different agents.

In contrast to unactivated macrophages, the mechanisms used by activated macrophages to recognize lipid-symmetric erythrocytes and apoptotic thymocytes appear to be similar. The phagocytosis of both types of target cells is inhibited by PS vesicles and by GlcNAc, and the effects of the two inhibitors on recognition of either target cell are not additive.[4] However, like unactivated macrophages, CD14 appears essential for recognition of lipid-symmetric erythrocytes since phagocytosis is completely abolished by PI-PLC treatment. In the case of thymocytes, both PS and GlcNAc reduce somewhat the activity persisting following PI-PLC treatment, consistent with the idea that PS vesicles and GlcNAc act on the same signal/receptor complement. That such different molecules are inhibitory again implies that the receptor and/or signal is complex.

The fact that PI-PLC treatment does not completely abolish recognition of apoptotic cells may simply reflect the incomplete removal of CD14 from the macrophage cell surface. If so, two observations require further consideration. First, why might incomplete removal completely abolish recognition of lipid-symmetric erythrocytes but not of apoptotic thymocytes for both unactivated and activated macrophages? One possibility is that erythrocytes lack signal components which are present on thymocytes, and which make recognition of apoptotic thymocytes possible even at low levels of CD14 expression on macrophages. Second, why is the residual activity not further reduced, in the case of unactivated macrophages, or only marginally further reduced, in the case of activated macrophages, by the presence of additional inhibitors? One possibility is that the residual activity following PI-PLC treatment reflects a separate recognition system which does not depend on the presence of CD14. LR-9 cells speak to this possibility; their complete inability to recognize and phagocytose apoptotic thymocytes suggests that an alternate, CD14-independent mechanism for recognition of apoptotic thymocytes is not present on the surface of unactivated macrophages.

At first, it might seem surprising that the phagocytosis of either lipid-symmetric or lipid-asymmetric erythrocytes by LR-9 cells was below the level of phagocytosis of lipid-asymmetric erythrocytes by parental J774 cells. Lipid-symmetric cells are prepared by lysis and resealing of erythrocytes in the presence of Ca^{2+}, which activates the scramblase leading to loss of asymmetry. Erythrocytes lysed and resealed in the presence of EGTA to chelate Ca^{2+} maintain their asymmetry, and thereby provide a control for the lysis and resealing process. When the phagocytosis of these lipid-asymmetric control erythrocytes and normal, intact erythrocytes are compared, the former are phagocytosed at a rate about 1.2–1.3 times higher than the latter.[14] If this slight increase in phagocytosis results from loss of asymmetry by a small fraction of cells in the lipid-asymmetric erythrocyte population, then it would be expected to be sensitive to the absence of CD14 in LR-9 cells.

Similarly, there is a likely explanation as to why the level of phagocytosis of either induced or uninduced thymocytes by LR-9 cells was below the level of phagocytosis of uninduced thymocytes by parental J774 cells. When thymocytes are incubated in culture in the absence of dexamethasone, a small fraction spontaneously undergo apoptosis, reflected by an increased number of cells

phagocytosed relative to fresh cultures of thymocytes.[4] Assuming that these spontaneously apoptotic cells present the same recognition signals as thymocytes induced to undergo apoptosis by dexamethasone treatment, their phagocytosis should be similarly sensitive to the absence of CD14.

The most remarkable finding presented here, however, is that the enhanced phagocytosis of either lipid-symmetric erythrocytes or apoptotic thymocytes is completely abolished in LR-9 cells. At face value, this result indicates that CD14 is necessary, but not necessarily sufficient, for recognition of lipid-symmetric erythrocytes and apoptotic thymocytes by these unactivated macrophages. Since CD14 is not a transmembrane protein, it likely must interact with other proteins to transduce a signal across the membrane, and may well interact with other proteins for recognition, consistent with the idea that recognition by both unactivated and activated macrophages is by a receptor complex. The identification of such proteins is a promising area for further work.

Finally, these studies suggest that CD14 or other proteins with which it associates may recognize PS alone or in association with other molecules on the surface of lipid-symmetric erythrocytes and apoptotic lymphocytes. However, whether these same mechanisms apply to apoptotic neutrophils as well is not yet clear since 61D3 mAb does not reliably inhibit phagocytosis of these cells by human monocyte-derived macrophages.[10,19] Since apoptotic neutrophils express PS on their surface, this discrepancy may reflect a deficiency of proteins with which PS must associate to generate a recognition signal on the neutrophil surface. It has recently been reported that human monocytic THP-1 cells transfected with CD14 display a twofold increase in low affinity binding of PS vesicles in the presence of LPS-binding protein.[20] Further studies will clearly be required to firmly establish whether or not CD14 is able to specifically recognize PS, alone or in association with other molecules, on the surface of apoptotic lymphocytes.

Materials and Methods

Materials

Bovine brain PS, FBS, dexamethasone, RGDS, β-glucan from barley, phosphatidylinositol-specific phospholipase C (PI-PLC), whole mouse IgG, mouse anti-human CD14 mAb (clone UCHM-1) and mouse anti-human glycophorin A,B mAb (clone E3) were purchased from Sigma Chemical Co. FITC-conjugated rat anti-mouse CD14 mAb (clone rmC5-3) and FITC-conjugated rat isotype control mAb (clone R3-34) were purchased from Pharmingen. Affi-gel protein A agarose was purchased from BioRad. Fluoresceinated latex beads (1 micron in diameter) were purchased from Polyscience.

Animals

Male CBA/J and BALB/c mice from Jackson Laboratories were maintained on food and water *ad libitum* in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Isolation and induction of apoptosis in thymocytes

Thymocytes were prepared from 4–6 week old CBA/J mice as previously described[4] and suspended at 10^7 cells/mL in DMEM containing 10% FBS. Apoptosis was induced by addition of 10^{-6} M dexamethasone, and cells were incubated for 6 h at 37°C in 5% CO_2. To monitor apoptosis, cells with hypodiploid DNA were enumerated. 10^6 cells were washed with PBS (7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, 137 mM NaCl, 10 mM KCl) and fixed by resuspending in equal volumes of PBS and 70% ethanol. Fixed cells were then centrifuged, resuspended in 200 μL of PBS, stained with propidium iodide at a final concentration of 18 μg/mL and analyzed by flow cytometry as described below.

Isolation and culture of primary macrophages

Inflammatory macrophages were elicited in the peritoneal cavity of 6–8 week old CBA/J mice by intraperitoneal injection of 1 mL of 3% Brewer's thioglycollate and harvested as previously described.[4] 6×10^5 cells in 150 μL of RPMI 1640 media containing 5% FBS were pipetted onto 18 mm bicarbonate-treated coverslips kept in 60 mm petri dishes. After a 2 h incubation at 37°C, nonadherent cells were removed by aspiration, and the media replaced with 150 μL/coverslip of fresh RPMI containing 5% FBS. The cells were cultured overnight at 37°C in 5% CO_2 before use in phagocytosis assays. Mouse bone marrow macrophages were prepared from CBA/J mice as previously described,[4] and 3×10^5 cells plated on coverslips 24 h prior to phagocytosis assays. Human monocyte-derived macrophages were prepared from fresh human venous blood obtained from volunteers according to institutional guidelines. The blood was collected into ice-cold PBS containing 10 U/mL of heparin, and centrifuged at $278 \times g$ for 5 min. The plasma was removed and heat-inactivated at 56°C for 30 min, and the buffy coat and top 10% of erythrocytes were removed and diluted 1:2 with PBS. Mononuclear cells in the suspension were separated by centrifugation on Ficoll-Paque™ and plated at a concentration of 4×10^6 cells/coverslip in 150 μL of RPMI 1640. After 2 h at 37°C, media and nonadherent cells were removed by aspiration and replaced with fresh RPMI 1640 containing 10% heat-inactivated autologous human serum. The medium was changed every 3 days, and the cells were used in phagocytosis assays after 7–10 days.

β-glucan activation of bone marrow macrophages

Bone marrow macrophages were resuspended at a concentration of 2×10^6 cells/mL in RPMI 1640 containing 100 μg/mL of β-glucan and incubated at 37°C for 72 h prior to use. 3×10^5 of the activated macrophages were plated onto coverslips for use in phagocytosis assays.

Cell culture

Cells of the J774A.1 mouse monocyte-derived macrophage cell line (American Type Culture Collection) and LR-9 cells (a gift of Drs. Tatsuji Nishihara and Masahiro Nishijima, The National Institute of Health, Shinjuku-ku, Tokyo) were cultured in RPMI 1640 media containing 10% FBS. Twenty-four hours prior to phagocytosis assays, coverslip cultures of 3×10^5 J774 or LR-9 cells were prepared. 61D3 hybridoma cells (a gift of Dr. Donald Capra, The University of Texas Southwestern Medical School, Dallas, TX, USA) were grown in RPMI 1640 containing 1% glutamine, 1% sodium pyruvate and 10% FBS.

PI-PLC treatment of macrophages

Medium on coverslips containing macrophages was removed by aspiration and replaced with 150 μL of fresh RPMI containing 200 mU/mL of PI-PLC. After 1 h at 37°C, the coverslips were washed three times with PBS and used immediately in phagocytosis assays.

Preparation of inhibitors

Lipid-symmetric and lipid-asymmetric erythrocytes were prepared as previously described.[18] Human erythrocytes, collected as described above, were centrifuged into a pellet and the supernatant removed. Four volumes of lysis buffer (1/10 PBS containing 0.1 mM EGTA, 1 mM $MgCl_2$, and 0.1% BSA, plus 1 mM $CaCl_2$ for lipid-symmetric erthrocytes 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 several times, then placed at 37°C. After 30 min, 100 volumes of PBS were added, the cells pelleted, washed three times with PBS and finally resuspended in PBS at a concentration of 15×10^6 cells/mL. PS vesicles were prepared as previously described.[4] 61D3 mAb was purified from ascites. Briefly, female BALB/c mice primed with 95% pristane were injected intraperitoneally with 2×10^6 61D3 hybridoma cells in 0.5 mL of PBS and sacrificed after 2 weeks. The recovered ascites fluid was centrifuged at $3000 \times g$ for 15 min, incubated at 37°C for 1 h and stored overnight at 4°C. After adjusting the ascites fluid to pH 9, it was passed over a protein A sepharose column equilibrated with binding buffer (3.2 M NaCl, 1.6 M glycine, pH 9). The column was washed with binding buffer until the O.D. at 280 nm returned to background, and bound antibody was then eluted with 100 mM citrate, pH 3.

Phagocytosis assays

Thymocytes (10^6) or erythrocytes (15×10^6) in 150 μL of RPMI 1640, with or without inhibitors, were overlayed onto triplet coverslip cultures of macrophages and coverslips incubated at 37°C in 5% CO_2. After 30 min, coverslips were washed three times with ice-cold PBS, fixed either with 1.8% formaldehyde (thymocytes) or acidic methanol (erythrocytes), stained with Diff-Quik (Baxter), and phagocytosed cells enumerated as previously described in detail.[4] PS vesicles were present at 7.5 nMol/150 μL; erythrocytes, when used as an inhibitor, were present at 15×10^6 cells; RGDS was added to a final concentration of 1 mM; ten μg of 61D3 or control UCHM-1 mAb was added per 150 μL. All phagocytosis experiments for which results are presented were performed at least twice on different days with similar results, with most experiments being performed three to five times with similar results.

Latex bead phagocytosis assays

Macrophages were transferred into a 12-well tissue culture plate and incubated overnight. Fluoresceinated latex beads were washed twice in RPMI, diluted 10 000-fold in RPMI, and added to macrophages that either had or had not been treated with PI-PLC. After 30 min at 37°C in 5% CO_2 the cultures were washed three times with RPMI and incubated with 5 mM EDTA in PBS to release the macrophages from the dish prior to analysis by flow cytometry.

Fc receptor-mediated phagocytosis assays

Human erythrocytes were collected as described above. 15×10^6 erythrocytes suspended in 150 μL of PBS were incubated with 2 μg of anti-glycophorin A,B mAb on ice for 15 min, washed twice with PBS and then overlayed onto coverslips of macrophages. To block Fc receptor-mediated phagocytosis, 50 μg of whole mouse IgG was added to each coverslip of macrophages, incubated at 4°C and then washed three times with PBS before overlaying with erythrocytes treated with antibody.

Immunofluorescent staining

To measure CD14 expression, 1×10^6 J774 or LR-9 cells were incubated with 1 μg of FITC-conjugated rat anti-mouse CD14 mAb, or 1 μg of FITC-conjugated rat isotype control mAb, in 100 μL of staining buffer (PBS containing 1% FBS). After 25 min on ice, cells were washed once in staining buffer and analyzed by flow cytometry. All samples contained the membrane impermeant dye propidium iodide at 10 μg/mL to gate out dead cells.

Flow cytometry

A minimum of 10 000 cells/sample was analyzed using an EPIC 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 thymocytes with propidium iodide. Antibody (FITC) staining was monitored through a 525 nm bandpass filter, and fluorescence of latex beads was monitored through a 575 nm bandpass filter.

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