Induction of caspase- and reactive oxygen species-independent phosphatidylserine externalization in primary human neutrophils: role in macrophage recognition and engulfment

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Abstract: Macrophage recognition and disposal of neutrophils are important steps in the resolution of inflammation. Externalization of phosphatidylserine (PS) on the cell surface serves as a common recognition signal for macrophages and is associated with the apoptosis program in neutrophils. Here, we report that macrophage-differentiated PLB-985 cells induce rapid, caspase-independent PS externalization in human neutrophils. A similar degree of PS externalization was seen when neutrophils were cocultured with gp91phox-deficient PLB-985 macrophages, thus demonstrating that macrophage-induced PS externalization was NADPH oxidase-independent. Macrophage-induced PS externalization required cell-to-cell contact and kinase activation and was shown to correlate with neutrophil degranulation. Of note, the degree of engulfment of such PS-positive neutrophils by activated human monocyte-derived macrophages was considerably lower than for neutrophils undergoing constitutive apoptosis, indicating that PS externalization alone is not sufficient for macrophage disposal of neutrophils. However, addition of recombinant milk fat globule epidermal growth factor 8, a PS-binding protein, restored engulfment of the macrophage-cocultured target cells. Finally, neutrophils undergoing spontaneous apoptosis but not macrophage-cocultured neutrophils displayed surface expression and release of annexin I, and the addition of N-t-Boc-Phe-D-Leu-Phe-D-Leu-Phe (Boc1), a formyl peptide receptor/lipoxin receptor antagonist, suppressed clearance of apoptotic neutrophils. Conditioned medium from apoptotic neutrophils also promoted the engulfment of macrophage-cocultured neutrophils, and Boc1 blocked this process. Taken together, these studies highlight a novel pathway of PS externalization in primary human neutrophils and also provide evidence for an auxiliary function of annexin I in macrophage clearance of neutrophils. J. Leukoc. Biol. 85: 427–437; 2009.

Key Words: annexin I · inflammation · phagocytosis

INTRODUCTION

The recognition and disposal of cells that have succumbed to apoptosis are essential for the maintenance of tissue homeostasis and play an important role in the resolution of inflammation [1]. Dysregulation of the process of macrophage clearance of apoptotic cell corpses may result in autoimmune and chronic inflammatory diseases. The disposal of apoptotic cells is finely regulated by a system of recognition signals and their corresponding receptors on macrophages and is facilitated by soluble serum proteins that act as bridging molecules between phagocytes and apoptotic cells [2].

Cells dying by apoptosis undergo a dramatic rearrangement of their membrane surface with a collapse of phospholipid asymmetry and externalization of phosphatidylserine (PS), an important “eat-me” signal, on the cell surface [3–5]. Externalized PS, in turn, interacts directly with macrophages and other phagocytosis-competent cells through specific PS receptors [6–8] or indirectly, via bridging molecules such as milk fat globule epidermal growth factor 8 (MFG-E8), a PS-binding protein that also binds to integrin receptors on phagocytes through its arginine-glycine-aspartate motif [9, 10]. PS-dependent uptake of apoptotic cells stimulates macrophage secretion of anti-inflammatory cytokines and suppresses the production of inflammatory mediators [11]

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of proinflammatory mediators [11–13], thus contributing to the safe and noninflammatory removal of dying cells. In addition, recent studies have indicated that proteins such as annexin I and calreticulin can serve as recognition signals for macrophages [14, 15]. The relative importance of lipid- versus protein-mediated uptake of apoptotic cells has not been clarified to date.

The fate of apoptotic neutrophils at the site of inflammation is important for our understanding of many disease processes [16]. Neutrophils have been shown to expose PS during apoptosis [17, 18], and our previous studies have demonstrated that the externalization of PS in neutrophils may occur through a caspase-dependent or reactive oxygen species (ROS)-dependent pathway [19, 20]. In addition, recent studies have revealed several different pathways of nonapoptotic PS externalization in various cell types including neutrophils [21–23]. Indeed, we recently reported that LPS-stimulated RAW264.7 macrophages can induce PS externalization in neighboring HL-60 cells in the absence of apoptosis through a novel route involving nitrosative stress-mediated inhibition of aminophospholipid translocation in the target cell [24]. The latter findings suggest that macrophage-induced nitrosative/oxidative stress could contribute to the resolution of inflammation. In this study, we have analyzed macrophage-induced PS externalization in peripheral blood neutrophils and studied the fate of these neutrophils upon subsequent cocultivation with activated human macrophages in the presence or absence of a PS-binding, bridging protein.

**Materials and Methods**

**Reagents**

PMA, 1α,25-dihydroxycholecalciferol (VitD3), diphenyleneiodonium (DPI), TAMRA, aspartate-glutamate-valine-aspartate-7-amino-4-methylcoumarin (DEVD-AMC), benzoylxyoxycarbonyl-valine-alanine-aspartate-fluoromethylketone (zVAD-fmk), staurosporine (STS), cytochalasin B (CB), phospho-L-serine (PLS), and MLCP, genistein, 1-(5-isouquinolinesulfonfonyl)-2-methylpiperezine dihydrochloride (H-7), and rottlerin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Merocyanine 540 (MC540) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). N-t-Boc-Phe-D-Leu-Phe (Boc) and human recombinant myeloperoxidase (hMPO) were purchased from MP Biomedicals (Ellkirk, France) and R&D Systems (Minneapolis, MN, USA), respectively.

**Neutrophil isolation and cell culture**

Peripheral blood neutrophils were prepared from buffy coats of healthy adult blood donors as described previously [19]. Neutrophils were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce differentiation into macrophage-like cells, PLB-985 cells were seeded at a density of 1.0 × 10⁶ cells/ml in 24-well tissue-culture plates and stimulated using a combination of 30 nM PMA and 200 nM VitD3 for 3 days [26]. The human monocytic leukemia cell line THP-1, obtained from the ATCC (Manassas, VA, USA), was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, 5.0 × 10⁻³ M β-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce differentiation into macrophage-like cells, 5.0 × 10⁶ cells/ml were cultured with 50 ng/ml M-CSF (R&D Systems) for 3–4 days in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**THP-1 and PLB-985 differentiation and cell culture**

The wild-type (WT) and X chromosome-linked chronic granulomatous disease (X-CGD) human promyelocytic PLB-985 cell lines (the generous gift of Prof. Mary Dinauer, Indiana University School of Medicine, Indianapolis, IN, USA) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce differentiation into macrophage-like cells, PLB-985 cells were seeded at a density of 5.0 × 10⁵ cells/ml in 24-well tissue-culture plates and stimulated using a combination of 30 nM PMA and 200 nM VitD3 for 3 days [26]. The human monocytic leukemia cell line THP-1, obtained from American Type Culture Collection (Manassas, VA, USA), was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, 5.0 × 10⁻³ M β-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce differentiation into macrophage-like cells, 5.0 × 10⁵ cells/ml were cultured with 150 nM PMA for 3 days.

**Monocyte/macrophage cell-surface markers**

Expression of cell-surface markers was analyzed by flow cytometry as described previously [27]. Briefly, adherent PLB-985 or THP-1 cells differentiated as described above were removed by trypsinization and incubated with FITC-conjugated anti-human CD11b mAb (IgG1), PE-conjugated anti-human CD14 mAb (IgG2a), or isotype-matched control antibodies (Sigma Chemical Co.) for 30 min at room temperature. Cells were then resuspended in 2% paraformaldehyde and analyzed on a FACSscan (Becton Dickson, San Jose, CA, USA). Ten thousand events were collected for each sample, and data were analyzed using CellQuest software (Becton Dickinson). Low fluorescence debris was gated out before analysis.

**PS exposure**

PS externalization was determined by flow cytometric detection of annexin V binding using the protocol in the annexin V-FITC apoptosis detection kit (Oncogene Research Products, Cambridge, MA, USA). Briefly, cells were costained with annexin V-FITC and propidium iodide (PI, 125 ng/ml) in 10 mM HEPES buffer prior to analysis on a FACSscan (Becton Dickson) equipped with a 488-nm argon laser. Ten thousand events were collected for each sample, and data were analyzed using CellQuest software. Cell debris and residual macrophages were gated out before analysis on the basis of forward- and side-scatter properties. In competition studies with annexin V-FITC and MPO, neutrophils were stained with annexin V-FITC and PI in the presence of 10, 100, or 200 μM hMPO. For costaining with anti-MPO antibodies, PE-conjugated annexin V (Calbiochem) was used according to instructions provided by the manufacturer.

**MC540 staining**

Detection of altered lipid packing in the plasma membrane was assessed with MC540, essentially as described previously [28]. Briefly, 1.0 × 10⁶ cells were resuspended in Heps-buffered saline (HBS), pH 7.3, with 0.1% BSA. Then, 10 μl MC540 was diluted to 0.1 mg/ml in HBS was added, and cells were incubated for 3 min at room temperature. HBS (900 μl) was then added to each sample, and cells were analyzed on a FACSscan (Becton Dickson).

**Anti-PS antibody staining**

To verify the annexin V data, PS exposure was also analyzed with specific anti-PS mAb. Neutrophils were fixed in 4% formaldehyde for 15 min on ice and then blocked in human serum for 30 min. Cells were resuspended in PBS containing 1% FBS and incubated for 30 min on ice with FITC-conjugated anti-human PS mAb or isotype-matched control antibody (Upstate Cell Signaling Solutions, Temecula, CA, USA). Cell-surface expression of PS was analyzed on a FACSscan (Becton Dickson) operating with CellQuest software.

**Caspase-3 like enzyme activity**

Cleavage of the fluorogenic peptide substrate DEVD-AMC was determined as described previously [19]. Briefly, cell lysates and fluorogenic substrate were
combined in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM DTT, 1.0×10⁻⁶ M Nonidet P-40, and 0.1% CHAPS), and real-time recordings of enzyme-catalyzed AMC release were obtained using a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden).

**Determination of nuclear morphology**

Nuclear apoptosis was determined as described before [24]. Briefly, cells were harvested and resuspended in 2% paraformaldehyde. CytoTax preparations were stained with 1 μg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Cells were scored using a fluorescence microscope (Nikon ECLIPSE TE2000-S, Nikon Corp., Kanagawa, Japan).

**Assessment of hypodiploid DNA content**

DNA content was assessed as reported previously [29]. In brief, 0.5 × 10⁶ cells were harvested and resuspended in staining solution containing 50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate in PBS. The percentage of cells displaying hypodiploid (sub-G1) DNA content was then determined using a FACScan (Becton Dickinson) operating with CellQuest software.

**CD31 staining**

Neutrophils were resuspended in PBS containing 1% BSA and incubated with FITC-conjugated anti-human CD31 mAb (Serotec, MorpHexSys UK, Kidlington, UK) or isotype-matched control (Sigma Chemical Co.) antibody for 1 h at room temperature. CD31 expression was analyzed on a FACScan (Becton Dickinson) using the CellQuest software.

**Annexin I staining**

To study the cell-surface expression of annexin I, neutrophils were resuspended in staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing rabbit anti-human annexin I antibodies (Zymed Laboratories, San Francisco, CA, USA) or isotype-matched, negative control antibodies (Dako Diagnostics, Glostrup, Denmark). Cells were then incubated with secondary FITC-conjugated swine anti-rabbit polyclonal antibodies (Dako Diagnostics), and samples were analyzed on a FACScan (Becton Dickinson) using the CellQuest software.

**ROS production**

Production of superoxide was assessed by oxidation of dihydroethidium (DHE) to ethidium, as reported previously [5]. Briefly, cells were incubated with 5 μM DHE (Molecular Probes, Leiden, The Netherlands) in RPMI-1640 medium for 30 min at 37°C and then resuspended in PBS and submitted to flow cytometric analysis using a FACScan (Becton Dickinson) operating with CellQuest software (Becton Dickinson). Cellular debris and necrotic cells were excluded based on forward- and side-scatter characteristics.

**Neutrophil degranulation**

Neutrophils cocultivated with PLB-985 macrophages were resuspended in PBS containing 1% BSA and incubated for 30 min with FITC-conjugated anti-human MPO mAb (Dako Diagnostics, Glostrup, Denmark) or isotype-matched control antibody (Sigma Chemical Co.) for 30 min at room temperature. Membrane expression of MPO was then analyzed using a FACScan (Becton Dickinson). As a positive control for neutrophil degranulation, cells were preincubated with CB (10 μg/ml) for 5 min and then stimulated with fMLP (10 μM) for 15 min.

**Cocultivation of normal neutrophils and PLB-985 macrophages**

PLB-985 WT or X-CGD macrophages were washed extensively before cocultivation to remove residual amounts of the differentiating agents. Neutrophils (prechilled or freshly isolated) were then added to PLB-985 cultures in 24-well tissue-culture plates at a ratio of 10:1. For some experiments, DPI (10 μM) or 1100W (100 μM) was added to PLB-985 macrophages at 30 min prior to cocultivation, whereas zVAD-fmk (10 μM) was added at the beginning of cocultivation of PLB-985 macrophages and neutrophils. In addition, for some studies, neutrophils were pretreated with DPI (10 μM), genistein (100 μM), H-7 (6 μM), or rottlerin (30 μM) for 30 min or with STS (2 μM) for 1 h and washed in PBS before cocultivation with PLB-985 macrophages. After cocultivation at 37°C, supernatants were collected at the indicated time-points and spun down at 1200 rpm for 5 min to obtain (nonengulfed) neutrophils for further analysis.

**Phagocytosis assay**

To determine the degree of HMDM engulfment of neutrophils, apoptotic or PLB-985-cocultured neutrophils were pretreated with TAMRA [30] and then added to M-CSF-activated HMDM at a ratio of 10:1. HMDM and target cells were then cocultured at 37°C for 1 h. For some experiments, HMDM were pretreated with 1 μg/ml WT tMFG-E8 or D69E mutant MFG-E8 for 30 min; for these experiments, the recombinant proteins were also present throughout the phagocytosis assay. In addition, to investigate the role of annexin I in the engulfment of PLB-985-cocultured neutrophils, HMDM were preincubated for 30 min with conditioned medium from neutrophils cultured in serum-free medium for 20 h as described previously [31]. Thereafter, macrophages were rinsed twice with PBS before addition of target cells. For blocking experiments, HMDM were preincubated in the presence or absence of PLS (1 mM) or Boc1 (50 μM) 30 min prior to the phagocytosis assay, and Boc1 was also present during coculture. Following cocultivation, nonengulfed target cells were washed off with several washes in cold PBS, and the remaining cells were fixed in 2% paraformaldehyde for 15 min and then stained with Hoechst 33342 (1 μg/ml). Phagocytosis was evaluated by counting macrophages in visual light and thereafter, counting macrophage-engulfed, TAMRA-labeled cells under UV illumination using an inverted Nikon ECLIPSE TE2000-S fluorescence microscope (Nikon Corp.) equipped with a DS-5M digital camera operating with NIS-Elements software (Nikon Instruments, Baalhoevedorp, The Netherlands). A minimum of 300 macrophages per experimental condition was analyzed. Data are reported as the percentage of HMDM positive for uptake of TAMRA-labeled target cells. The phagocytosis index, i.e., the percentage of macrophages that had ingested one versus greater than or equal to two target cells, was also determined.

**MFG-E8 production**

tMFG-E8 and D69E mutant proteins were produced as described previously [9]. In brief, expression plasmids of FLAG-tagged MFG-E8 and mutant MFG-E8 (the generous gift of Prof. Shigekazu Nagata, Kyoto University, Kyoto, Japan) were introduced into human 293T cells using the LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA, USA), and the proteins secreted into the medium were purified using anti-Flag M2 affinity gel (Sigma Chemical Co.). To examine the purity of the proteins, they were subjected to SDS-PAGE and Western blotting using anti-FLAG antibody (Sigma Chemical Co.).

**Collection of conditioned medium**

Following cocultivation of PLB-985 macrophages and neutrophils, supernatants (conditioned medium) were collected and spun down at 1200 rpm and 3000 rpm for 5 min, respectively, to remove cells and cell debris. Naïve neutrophils were then incubated at 37°C for the indicated time-points in conditioned medium or standard (fresh) RPMI-1640 medium. Neutrophils were then harvested, and PS externalization was determined as described above.

**Western blot analysis**

Supernatants from cocultures were harvested and spun down at 1000 rpm for 5 min followed by centrifugation at 13,000 rpm for 3 min. Equal volumes (30 μL) of each sample were then resolved by electrophoresis on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA), which were blocked with 5% milk in 0.1% Tween-20PBS and then incubated with the rabbit polyclonal antiannexin I antibody (Zymed Laboratories). After washing with PBS-Tween, membranes were incubated with HRP-conjugated anti-rabbit secondary antibody (Dako Diagnostics). Protein bands were visualized with ECL reagents according to the manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK).

**Statistics**

The results are expressed as mean values ± SEM. Samples were analyzed by Student’s t-test, and differences between mean values were considered significant when P < 0.05.
RESULTS

VitD₃ and PMA induce macrophage differentiation of the PLB-985 cell line

We have reported previously that NADPH oxidase-generated ROS are critical for PS externalization in activated human neutrophils [19]. To test the hypothesis that a functional NADPH oxidase in macrophages is required for macrophage-induced PS externalization in neutrophils, we used the PLB-985 human promyelocytic cell line [32] and a variant of the same cell line in which the gene encoding gp91phox, an essential cytosolic component of the NADPH oxidase, has been disrupted by homologous recombination [33]. PLB-985 cells can be induced to undergo granulocytic or monocytic differentiation, depending on the differentiating agents. In the present study, we exposed PLB-985 WT and X-CGD cells to PMA or VitD₃ alone or to a combination of both agents and determined the degree of differentiation by assessment of plastic adherence, morphological features, and detection of phenotypic cell-surface markers. We found that a combination of VitD₃ (200 nM) and PMA (30 nM) was required for successful differentiation of PLB-985 cells to monocyte/macrophage-like cells based on the abovementioned criteria, yielding a phenotype comparable with PMA-treated (150 nM) THP-1 cells, a well-established macrophage-like cell model (Supplemental Fig. 1A). Moreover, VitD₃ and PMA induced CD11b expression (Supplemental Fig. 1B) but not CD14 expression (data not shown) on the surface of PLB-985 cells. Importantly, a similar degree of differentiation was observed in PLB-985 WT and X-CGD cells.

PLB-985 macrophages induce PS externalization on the surface of normal neutrophils

Recent studies have shown that macrophages may play a role in PS externalization on the surface of neighboring target cells [24]. To investigate this phenomenon further, we added normal, prechilled neutrophils [25] to cultures of PLB-985 WT macrophages. Nonengulfed neutrophils were then recovered from the cocultures at the indicated time-points, and the degree of PS externalization was determined using the annexin V-FITC/PI protocol. Neutrophils maintained in culture for the same period in the absence of macrophages were used for comparison. As shown in Figure 1A, macrophage-induced PS externalization was seen as early as 30 min after cocultivation and increased in a time-dependent manner, whereas neutrophils incubated alone displayed minimal (<10%) PS exposure at the same time-points (Fig. 1, A and B). In addition, we observed similar findings when neutrophils were cocultured with THP-1 macrophages (Fig. 1C). However, undifferentiated PLB-985 or THP-1 cells did not induce any PS exposure (Fig. 1C). After 2 h, most neutrophils were strongly attached to macrophages; therefore, isolation of neutrophils from the co-

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Fig. 1. PS externalization on the surface of neutrophils upon cocultivation with PLB-985 macrophages. (A) Neutrophils were cocultured with PLB-985 macrophages or incubated alone at 37°C for the indicated time-points. At the end of incubation, nonadherent cells were harvested, and cells were costained with annexin V-FITC and PI as described in Materials and Methods. (B) Quantification of the percentage of PI-negative, annexin V-positive cells. Data are derived from at least five independent experiments using different donors and are displayed as mean values ± SEM. (C) TAMRA-stained neutrophils were cocultured for 1 h with undifferentiated or differentiated PLB-985 or THP-1 cells or incubated alone at 37°C. At the end of incubation, nonadherent cells were harvested, and cells were stained with annexin V-FITC. Only TAMRA-positive cells were included in the analysis by gating on this population. Quantification of the percentage of annexin V-positive cells is shown. Data are derived from at least three independent experiments using different donors and are displayed as mean values ± SEM.
cultures for assessment of PS externalization was not possible beyond this time-point. Of note, cultivation of neutrophils in the presence of PMA (30 nM) and VitD3 (200 nM) for 2 h did not induce PS externalization, thus excluding a potential effect of the macrophage-differentiating agents on neutrophils (data not shown).

To verify our results using annexin V-FITC, we adopted several additional approaches. First, we monitored alterations in lipid packing in the plasma membrane with MC540. Lipid asymmetry is known to be accompanied by altered packing of the membrane, which enhances MC540 fluorescence [34]. Supplemental Figure 2A shows that the results obtained using MC540 as a probe are comparable with the annexin V-FITC data, indicating that the observed PS exposure is not a result of nonspecific annexin V binding. Furthermore, as seen in Supplemental Figure 2B, annexin V-FITC staining of macrophage-cocultured neutrophils correlates well with specific anti-PS mAb staining. However, it is noted that anti-PS antibodies do not seem to detect secondary necrotic (PI-positive) cells. We also performed control experiments to exclude the possibility that annexin V-FITC is binding to MPO on the surface of degranulated (macrophage-induced) neutrophils (Supplemental Fig. 2, C and D). Together, our studies demonstrate that macrophage-differentiated cells can trigger PS externalization in primary human neutrophils.

**PLB-985 macrophage-induced PS externalization is caspase-independent**

PS externalization may be associated with or uncoupled from caspase activation during apoptosis [35, 36]. To test whether PLB-985 macrophage-induced PS exposure in neutrophils is linked to the induction of the common apoptotic pathway, neutrophils and PLB-985 WT macrophages were cocultured in the presence or absence of the broad-spectrum caspase inhibitor zVAD-fmk (10 μM). Neutrophils undergoing constitutive apoptosis following prolonged (22 h) culture were also examined. Cocultivation of neutrophils with PLB-985 WT macrophages for 2 h induced a modest increase in apoptosis, in comparison with neutrophils maintained in culture for 22 h (Supplemental Fig. 3, A and B). However, PS exposure in neutrophils cocultivated with PLB-985 macrophages was not affected by zVAD-fmk, and caspase inhibition partially blocked PS exposure in neutrophils undergoing spontaneous apoptosis (Supplemental Fig. 3C), in line with our previous studies [19]. Moreover, no significant differences in terms of caspase-3 activation were seen between neutrophils cocultivated with macrophages and control neutrophils (data not shown). These results thus suggest that macrophage-induced PS in neutrophils is caspase-independent.

**Macrophage-induced PS externalization is NADPH oxidase- and inducible NO synthase (iNOS)-independent**

NADPH oxidase-generated ROS have been shown to contribute to PS externalization in activated neutrophils [19]. Moreover, recent studies have demonstrated that macrophage-induced nitrosative stress can induce PS exposure in neighboring target cells [24]. To investigate whether macrophage-induced PS exposure is NADPH oxidase-dependent, PLB-985 WT macrophages were pretreated with DPI, a nonspecific inhibitor of the NADPH oxidase prior to cocultivation with neutrophils. To complement these inhibitor studies, neutrophils were also cocultivated with PLB-985 X-CGD macrophages harboring a nonfunctional NADPH oxidase. However, PS externalization on the surface of neutrophils was unaffected under these conditions (Fig. 2A). Of note, cocultivation of neutrophils with PLB-985 WT macrophages stimulated neutrophils to produce ROS, and this was blocked effectively upon addition of DPI to cocultures of PLB-985 macrophages and neutrophils (Supplemental Fig. 4A). However, DPI failed to affect PS exposure under these conditions (Supplemental Fig. 4B). To test whether nitrosative stress was involved, we added 1400W, a selective inhibitor of iNOS to PLB-985 macrophages. However, as seen

![Fig. 2. NADPH oxidase- and iNOS-independent PS externalization in neutrophils cocultivated with PLB-985 macrophages. (A) PLB-985 WT macrophages were pretreated with DPI (10 μM) for 30 min. Neutrophils were then cocultured with PLB-985 WT macrophages for 1 h. Neutrophils cocultivated for 1 h with PLB-985 X-CGD macrophages lacking a functional NADPH oxidase were also included. PS exposure in cocultivated neutrophils was then determined. Data shown are mean values ± SEM (n=3). (B) PLB-985 WT macrophages were pretreated with the iNOS inhibitor, 1400W (100 μM), for 1 h, and neutrophils were then added to PLB-985 macrophages. PS externalization in neutrophils was determined following 1 h of coculture. Data are reported as mean values ± SEM (n=3).](image-url)
in Figure 2B, there were no significant differences in PS exposure in the presence or absence of the iNOS inhibitor.

**PLB-985 macrophage-induced PS externalization in neutrophils is kinase-dependent**

STS, a broad-spectrum protein kinase C (PKC) inhibitor, has been shown previously to inhibit PS externalization in pre-chilled neutrophils undergoing massive synchronous apoptosis [25]. To determine whether PKC plays a role in PLB-985 macrophage-induced PS externalization, we pretreated neutrophils with STS before adding these cells to PLB-985 macrophages. STS completely blocked the macrophage-induced externalization of PS (Fig. 3A). Moreover, we tested additional kinase inhibitors with different specificities including genistein, H-7, and rottlerin. Genistein (100 μM) significantly decreased macrophage-induced externalization of PS, whereas the selective PKC inhibitors, H-7 and rottlerin, were ineffective (Fig. 3A), indicating that the activation of PKC is not solely responsible for this phenomenon. Indeed, genistein and STS are also well-known inhibitors of tyrosine kinases [37]. We also noted that STS prevented the cell-to-cell interaction between neutrophils and PLB-985 macrophages (data not shown). To further confirm that PLB-985 macrophage-induced PS exposure in neutrophils requires cell-to-cell contact, we collected the cell-free supernatants upon cocultivation of PLB-985 macrophages and normal neutrophils and added the supernatants to naive neutrophils. However, the conditioned medium failed to promote PS exposure when compared with fresh medium (data not shown), implying that the induction of PS exposure in neutrophils by PLB-985 macrophages requires interaction between the two cell types and is not mediated by soluble signals.

**PLB-985 macrophages trigger kinase-dependent neutrophil degranulation**

The requirement for cell-to-cell contact between macrophages and neutrophils is suggestive of a role for adhesion molecules, which could transmit outside-in signaling in neutrophils. Importantly, the engagement of adhesion molecules on the surface of neutrophils can lead to the release of neutrophil granule contents [38, 39]. To test whether macrophage-induced PS externalization is associated with degranulation, we determined the degree of cell-surface expression of MPO, a component of azurophilic neutrophil granules following cocultivation of neutrophils and PLB-985 macrophages. Cocultivation with macrophages triggered a pronounced degranulation, as assessed by flow cytometry (Fig. 3B). Moreover, this event was blocked almost completely by STS. As a positive control for degranulation, the chemotactic peptide, fMLP was used in combination with the priming agent, CB.

**Neutrophils with macrophage-induced PS are poorly engulfed compared with neutrophils undergoing spontaneous apoptosis**

PS externalization on the surface of apoptotic cells is believed to serve as an important recognition signal for macrophage clearance of apoptotic cells [1]. PLB-985 macrophages are much less proficient at phagocytosis than HMDM (data not shown). Therefore, to test whether externalized PS is sufficient for engulfment of PLB-985-cocultivated neutrophils, we added the cocultivated neutrophils to activated HMDM. Importantly, the percentage of PS-positive neutrophils following cocultivation with PLB-985 WT macrophages was equal to that of neutrophils undergoing spontaneous apoptosis (Fig. 4A).

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*Fig. 3. Macrophage-induced PS externalization and degranulation is kinase-dependent. (A) Neutrophils were pretreated with STS (2 μM) for 1 h or with genistein (100 μM), H-7 (6 μM), or rottlerin (30 μM) for 30 min and then added to PLB-985 WT macrophages and cocultured for 2 h. Neutrophils maintained in culture in the absence of PLB-985 macrophages were included as a control. The percentages of annexin V-FITC-positive cells are shown as mean values ± SEM (n=5). Spont. apopt. Spontaneous apoptosis. (B) Neutrophils obtained from cocultivation with PLB-985 macrophages were incubated with FITC-conjugated anti-MPO antibodies or isotype-matched control antibodies for assessment of neutrophil degranulation. Neutrophils treated with the combination of fMLP (10 μM) and CB (10 μg/ml) served as a positive control. Data are displayed as the mean fluorescence intensity (MFI) of three independent experiments (mean±SEM).*

Moreover, the MFI of annexin V-FITC-positive cells was similar in both cases, suggesting that the amount of PS externalization was also comparable (data not shown). However, phagocytosis of PLB-985-cocultivated neutrophils by HMDM was not as efficient as for neutrophils undergoing spontaneous apoptosis (Fig. 4B). To determine whether engulfment of apoptotic neutrophils was PS-dependent, the inhibitor of PS-mediated uptake, PLS, was added to cocultures of apoptotic neutrophils and HMDM, and the percentage of phagocytosis-positive macrophages was calculated. As seen in Figure 4C, administration of PLS resulted in significant inhibition of phagocytosis, which is in accordance with previous studies [40].

Engulfment of apoptotic cells may also depend, in part, on the disabling or down-regulation of “don’t-eat-me” signals, including CD31 (also known as platelet-endothelial cell adhesion molecule-1) [41]. We therefore asked whether PLB-985-cocultivated neutrophils failed to down-regulate CD31 expression. However, neutrophils undergoing spontaneous apoptosis (22 h) and neutrophils collected upon cocultivation with PLB-985 macrophages (2 h) displayed a similar loss of reactivity with specific anti-CD31 antibodies (data not shown). Taken together, these results show that PLB-985 macrophage-induced PS externalization in neutrophils may not be sufficient for clearance of these cells by activated human macrophages and suggest therefore that surface expression and/or release of additional recognition ligand(s) are lacking in this model.

MFG-E8 restores HMDM clearance of neutrophils with PLB-985 macrophage-induced PS externalization

Externalized PS may be recognized directly via PS receptors on the macrophage surface or indirectly via so-called bridging molecules [2]. MFG-E8 has been shown to facilitate ingestion of PS-positive target cells by activated human macrophages [30]. We therefore hypothesized that MFG-E8 should bind to externalized PS on PLB-985-cocultivated neutrophils and promote clearance of these neutrophils by activated human macrophages. To this end, MFG-E8 or the D89E mutant form of MFG-E8 were added to cocultures of PLB-985-induced neutrophils and HMDM. The presence of MFG-E8 increased the phagocytosis of neutrophils significantly by HMDM, almost to the same level as the phagocytosis of neutrophils undergoing spontaneous apoptosis (Fig. 5A). The D89E mutant, however, did not affect HMDM uptake of neutrophils. MFG-E8 also increased the phagocytic index (i.e., percentage of macrophages that ingested more than one neutrophil; Fig. 5, B and C), in line with previous studies using apoptotic thymocytes as target cells [9].

Annexin I is involved in the clearance of primary neutrophils with macrophage-induced PS

Moreover, the release of annexin I (a component of gelatinase

Proteins such as annexin I and its peptide derivatives have been shown to facilitate uptake of cells undergoing spontaneous apoptosis [31]. Interestingly, we observed that neutrophils undergoing spontaneous apoptosis exposed significant levels of annexin I on the cell surface, whereas neutrophils cocultured for 2 h with PLB-985 macrophages failed to do so (Fig. 6A). Moreover, the release of annexin I (a component of gelatinase

Fig. 4. Phagocytosis of primary human PLB-985-cocultivated neutrophils by activated HMDM. (A) Comparison of PS externalization in neutrophils following cocultivation with (co-culture) or without (control) PLB-985 WT macrophages for 2 h versus neutrophils undergoing spontaneous apoptosis. Data are shown as mean values ± SEM (n=3). (B) Phagocytosis of neutrophils maintained in culture as indicated above. Neutrophils were added to M-CSF-activated HMDM and cocultured for 1 h at 37°C prior to assessment of the degree of phagocytosis. Data are shown as mean values ± SEM (n=3). (C) HMDM engulfment of control neutrophils or neutrophils undergoing spontaneous apoptosis was monitored in the presence or absence of PLS (1 mM). Data are reported as mean values ± SEM (n=3).
neutrophil granules) into the supernatant was considerably lower in supernatants from neutrophils cocultured with PLB-985 macrophages than for spontaneous apoptotic neutrophils (Fig. 6B). Administration of the formyl peptide receptor/lipoxin receptor antagonist, Boc1 [42], partially decreased uptake of apoptotic neutrophils by HMDM, indicating that annexin I is involved in the engulfment of these cells (Fig. 6C). Moreover, when preincubating macrophages with conditioned medium from apoptotic neutrophils, which is rich in annexin I, we observed a twofold increase in phagocytosis of PLB-985-cocultured neutrophils, and this enhancement of cell clearance was blocked by the addition of Boc1 (Fig. 6D). Together, these results show that annexin I secretion plays an important role in macrophage clearance of neutrophils.

DISCUSSION

Ten years ago, we described two distinct pathways of PS externalization in neutrophils: a caspase-dependent pathway that appears to operate during constitutive apoptosis and a ROS-dependent pathway that functions in activated neutrophils in which caspase activation is disabled [19]. Here, we describe a novel pathway of PS externalization in neutrophils that is neither caspase- nor ROS-dependent but instead, appears to be associated with macrophage-induced neutrophil degranulation. We also demonstrate that PS externalization alone may not be sufficient for engulfment of neutrophils by activated HMDM and provide evidence for an auxiliary role of neutrophil-derived annexin I in this process.

We initially hypothesized that a functional NADPH oxidase in macrophages is required for macrophage-induced PS externalization in neutrophils, and for this reason, we used the PLB-985 human promyelocytic cell line and a variant of the same cell line in which the gene encoding gp91phox has been disrupted by homologous recombination [33]. However, both variants of this cell line were capable of triggering PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation. Our recent findings have provided evidence that macrophage-induced nitrosative stress can trigger PS externalization in neutrophils, thus indicating that PS externalization in this model is independent of NADPH oxidase activation.

Fig. 5. MFG-E8 enhances phagocytosis of neutrophils with PLB-985 macrophage-induced PS externalization. (A) HMDM were pretreated with MFG-E8 (WT) or D89E MFG-E8 mutant protein (1 μg/ml) for 30 min, and the recombinant proteins were added again at the time of cocultivation. Neutrophils were cocultured with HMDM for 1 h, and the percentages of phagocytosis-positive macrophages were determined. Neutrophils undergoing spontaneous apoptosis and neutrophils maintained in culture for 2 h in the absence of PLB-985 macrophages were included for comparison. Data are derived from four independent experiments and are reported as mean values ± SEM. (B) Cocultivation of neutrophils and HMDM was performed as above, and the percentages of macrophages that carried one or greater than or equal to two target cells in the presence or absence of MFG-E8 (1 μg/ml) were determined. Data shown are mean values ± SEM (n=3). (C) Representative images showing HMDM engulfment of TAMRA-labeled target neutrophils (red) in the presence or absence of MFG-E8 (1 μg/ml). Hoechst 33342 (blue) was used for visualization of cell nuclei. Arrows indicate macrophages that have ingested more than one target cell. Original magnification, ×40.
to certain types of macrophages or macrophage-like cell lines, including PLB-985, THP-1, and RAW264.7 macrophages. Notwithstanding, the current system provides a convenient model in which to test the importance of PS externalization for macrophage clearance of primary human neutrophils and to gauge the role of other recognition or eat-me signals in this process.

PS externalization has been shown to occur during neutrophil apoptosis [17, 18] and is believed to be a common recognition signal for macrophages. However, the present studies demonstrate that PLB-985-cocultivated neutrophils are poorly engulfed by activated human macrophages, despite the occurrence of high percentages of PS-expressing cells. Indeed, the level of PS externalization was comparable in macrophage-cocultivated neutrophils and neutrophils undergoing spontaneous apoptosis, indicating that the deficiency in cell clearance is not a result of subthreshold levels of PS [43]. This suggests that PS externalization alone is not sufficient for macrophage clearance by macrophages, an observation that concurs with the recent studies reported by Guzik et al. [44], who found that bacterial or host proteases affected uptake of neutrophils without having an effect on cell-surface PS presentation. Indeed, fMLP-induced degranulation and externalization of PS in neutrophils have been reported previously, and these neutrophils were not recognized by macrophages [45]. Importantly, however, we found that clearance of macrophage-cocultivated neutrophils by activated HMDM could be restored upon administration of a PS-binding bridging protein, MFG-E8, but not by the mutant form of this protein. Moreover, the addition of PLS to cocultures of apoptotic neutrophils and HMDM partially inhibited uptake, indicating that PS externalization is required for macrophage engulfment. Based on these studies, we conclude that PS externalization is necessary but not sufficient for macrophage clearance of neutrophils. Our data also show that PS externalization may promote efficient cell clearance in the presence of opsonizing molecules such as MFG-E8 that bind to PS.

Annexin I externalization on the surface of apoptotic cells has been suggested to serve as a recognition signal for non-professional phagocytes (HUVEC) [14], and recent studies have shown that certain apoptotic cells may also secrete annexin I and peptide derivatives, thereby facilitating uptake by professional phagocytes (macrophages) [31, 42]. We observed that neutrophils undergoing constitutive apoptosis displayed significant levels of annexin I on the cell surface and secreted annexin I into the culture medium, whereas macrophage-cocul-

**Fig. 6.** Annexin I is involved in the phagocytosis of neutrophils with PLB-985 macrophage-induced PS externalization. (A) Annexin I externalization in neutrophils undergoing spontaneous apoptosis versus neutrophils cocultured with PLB-985 macrophages or cultured alone (control) was analyzed by flow cytometry using specific antiannexin I antibodies. The percentages of annexin I-positive cells with background subtracted (isotype-matched control antibodies) are shown. Data are reported as mean values ± SEM (n=3). (B) Secretion of annexin I into the culture medium was analyzed by Western blotting. Supernatants from control neutrophils cultured alone for 2 h or neutrophils maintained in culture for 22 h (spontaneous apoptosis) and supernatants from cocultures of PLB-985 macrophages and neutrophils (“co-culture 2 h”) or neutrophils cocultured with PLB-985 macrophages for 2 h and then incubated alone in fresh medium for 2 h (“co-culture 2 h + 2 h”) were analyzed using specific antiannexin I antibodies. A comparison of the latter two samples reveals that cocultured neutrophils fail to secrete annexin I, whereas PLB-985 macrophages may release annexin I following interaction with neutrophils. For comparison, immunoblotting of total cell lysates of the corresponding samples is also shown. Results are representative of several independent experiments. (C) Phagocytosis of apoptotic neutrophil target cells (in vitro culture for 22 h) by HMDM in the presence or absence of the formyl peptide receptor/lipoxin receptor antagonist, Boc1 (50 μM). (D) Phagocytosis of neutrophils cocultured with PLB-985 macrophages by HMDM pre-treated with conditioned medium from apoptotic neutrophils in the presence or absence of Boc1 (50 μM). Data shown in Panels C and D are mean values ± SEM (n=4).
tured neutrophils failed to do so. This indicates that the translocation of annexin I, a known PS-binding protein, does not necessarily accompany PS externalization [14]. Furthermore, we found that Boc1, a formyl peptide receptor/lipoxin receptor antagonist, suppresses uptake of apoptotic neutrophils, suggestive of a role for annexin I in the clearance of these cells [42]. Importantly, when conditioned medium from apoptotic neutrophils was added to macrophage-cocultured neutrophils, the subsequent engulfment of these cells was enhanced, and this process was also blocked by addition of Boc1. In sum, our studies show that annexin I plays an important role in macrophage clearance of primary human neutrophils and that the externalization of PS and secretion of annexin I contribute to efficient engulfment of these cells.

These studies have thus provided information about the role of PS externalization for macrophage clearance of neutrophils and suggest that a growing number of signals, including various lipid species and secreted proteins, are involved in the disposal of neutrophils by macrophages [1]. Further studies are needed to dissect the novel pathway of PS externalization described in the present report. We thus observed that macrophage-induced PS externalization in nonapoptotic (preapoptotic) neutrophils is dependent on kinase activation, and based on our inhibitor studies, tyrosine kinases are likely to be involved, but we can only speculate on the underlying mechanism at this point. One possibility is that cell-to-cell contact between neutrophils and certain classes of macrophages activates adhesion molecules, leading to degranalation of neutrophils with concomitant exposure of PS on the cell surface. For comparison, previous studies have documented degranulation-associated PS externalization in human and murine mast cells [46]. PS-dependent ingestion of apoptotic cells has been shown to promote macrophage secretion of anti-inflammatory cytokines and the resolution of acute inflammation [12]. Indeed, cell-to-cell contact between PS-positive target cells and activated macrophages appears to be sufficient to induce profound alterations in the pattern of cytokine secretion [13]. It will be of interest to clarify the role of macrophage-induced PS externalization in preapoptotic neutrophils for the promotion or resolution of inflammation.

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