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Modulation of Phagocytosis of Apoptotic Neutrophils by Supernatant from Dexamethasone-Treated Macrophages and Annexin-Derived Peptide Ac2–26

Paola Maderna,2* Simon Yona,† Mauro Perretti,† and Catherine Godson*

Phagocytic clearance of apoptotic leukocytes plays an important role in the resolution of inflammation. The glucocorticoid-inducible protein annexin 1 and annexin 1-derived peptides show potent anti-inflammatory responses in acute and chronic inflammation. In this study, we report that the annexin 1-derived peptide (Ac2–26) significantly stimulates nonphlogistic phagocytosis of apoptotic polymorphonuclear leukocytes (PMNs) by human monocyte-derived macrophages (Mφ). Peptide Ac2–26-stimulated phagocytosis is accompanied by rearrangement of the Mφ actin cytoskeleton. To investigate the potential role of endogenous annexin on clearance of apoptotic cells, Mφ were cultured for 5 days in the presence of dexamethasone. Supernatants collected from dexamethasone-treated Mφ significantly enhanced the ability of naïve Mφ to engulf apoptotic PMNs. This effect was blocked by an annexin blocking Ab, by immunodepletion of the supernatants, and by the formyl peptide receptor/lipoxin receptor antagonist Boc1. In addition, we show that bone marrow-derived Mφ from annexin 1-null mice present a 40% decreased phagocytosis of apoptotic PMNs compared with cells taken from littermate controls. In conclusion, these results emphasize the pivotal role of annexin 1 as mediator for clearance of apoptotic cells and expand its potential therapeutic role in controlling inflammatory diseases. The Journal of Immunology, 2005, 174: 3727–3733.

It is increasingly appreciated that macrophage (Mφ) phagocytosis of apoptotic cells plays an important role in the resolution of inflammation protecting tissue from harmful exposure to the inflammatory and immunogenic contents of dying cells (1–3). Should cells die by necrosis and disintegrate in situ, release of their contents may exacerbate the local inflammatory response and trigger further leukocyte influx. In addition to removing cells before they undergo lysis, it is proposed that ingestion of apoptotic cells results in potent anti-inflammatory and immunosuppressive effects through the production of anti-inflammatory cytokines such as TGF-β, IL-10, IL-13, IL-1Ra, and the suppression of release of proinflammatory mediators (4, 5). In this context, endogenous and exogenous regulators of this process may have therapeutic potential (6). We have previously demonstrated that endogenously produced lipoxin (LX), LXA4, and LXB4, and stable LX synthetic analogs can promote nonphlogistic phagocytosis of apoptotic polymorphonuclear leukocytes (PMNs) by human monocyte-derived Mφ in vitro (7) and in vivo (8). This prophagocytic effect seems to be coupled to LX-mediated alterations of the Mφ actin cytoskeleton (9) and further expands on the potential proresolving bioactions of LXs (10, 11).

In this study, we have investigated whether a peptide mimetic of annexin 1, Ac2–26, a recently described agonist for the LXA4 receptor (ALXR) (12, 13) can promote phagocytosis of apoptotic leukocytes. Annexin 1 is a glucocorticoid-inducible protein (14) that mediates many of the anti-inflammatory actions of glucocorticoids in models of acute (15) and chronic (16) inflammation including modulation of leukocyte trafficking (17, 18), ischemic damage (19), pain (20), and fever (21). Evidence for a functional link between annexin 1 and apoptosis has emerged (22, 23). On one hand, exogenous annexin 1 increases the rate of spontaneous PMN apoptosis (22), whereas endogenous annexin 1 is exported from the cytosol to the plasma membrane of apoptotic cells where it colocalizes with phosphatidylserine (PS), suggesting a role for annexin 1 in recognition of cell for phagocytic clearance (23). More recently, it has been reported that phagocytosis of apoptotic lymphocytes by Mφ was inhibited by pretreatment of either target cells or phagocytes with an Ab to annexin 1, suggesting that annexin serves as both ligand and receptor in promoting phagocytosis (24).

In this study, we demonstrate that the peptide Ac2–26 promotes phagocytosis of apoptotic PMNs by human Mφ. We have investigated the underlying mechanisms, and we have explored whether the previously reported effect of dexamethasone (Dex) to enhance phagocytosis of apoptotic leukocytes (25, 26) could be attributed to Dex induction of annexin 1 expression. Furthermore, we report that bone marrow-derived Mφ from annexin 1-null mice are defective in clearance of apoptotic cells.

Materials and Methods

Materials

LXA4 was obtained from Biomol Research Laboratories. The annexin 1 mimetic peptide Ac2–26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) was prepared by the Advance Biotechnology Centre (The Charing Cross and Westminster Medical School, London, U.K.) by using solid-phase
stepwise synthesis. Purity was >90% as assessed by HPLC and capillary electrophoresis (data supplied by manufacturer). A polyclonal sheep serum raised against the annexin 1-derived peptide Ac2–26 (LCPS1) that neutralizes the action of Dex and annexin 1 (27), was used as described by Perretti et al. (27). Adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP) was obtained from Calbiochem (Nottingham, U.K.). 8-Bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and Dex were obtained from Sigma-\textregistered Aldrich. The antagonist Boc1 (N-Boc-Phe-o-Leu-Phe-o-Leu-Phe) that blocks both the formyl-peptide receptor (FPR) and ALXR activation (13) was purchased from MP Biomedicals. Oregon Green phalloidin was from Molecular Probes. The TGF-\beta1 and IL-8 human ELISA kits were obtained from R&D Systems Europe. A rabbit anti-human annexin 1 Ab was from Zymed (Cambridge Biosciences).

Reagents were dissolved in DMSO or ethanol and further diluted in medium (final concentration, <0.1%). Equivalent concentrations of DMSO or ethanol were used as vehicle controls.

**Human leukocyte isolation**

Human PMNs and monocytes were isolated from peripheral venous blood drawn from healthy volunteers, after informed written consent, as previously described (7, 9). Briefly, mononuclear cells were separated by centrifugation on Ficoll-Paque (Pharmacia) and plated at 2 × 10^8 cells/ml in RPMI 1640 (BioWhittaker) supplemented with 10% autologous serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 \mu g/ml streptomycin (Invitrogen Life Technologies). Lymphocytes were removed after 1-h culture, and Mφ were obtained by further culturing monocytes for 7 days.

For preparation of supernatant from Dex-treated Mφ, monocytes prepared as above were cultured for 5 days in RPMI 1640 supplemented with 10% serum in the presence or absence of 1 μM Dex. The supernatants were collected, centrifuged at 14,000 rpm for 10 min, and stored at −20°C.

PMNs, after dextran sedimentation (Dextran T500; Pharmacia) and hypotonic lysis of red cells, were suspended at 4 × 10^6 cells/ml. Potassium-stimulated PMNs were harvested (400 × g; 10 min). Cells were then resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% FCS, and 20% L929 conditioned medium. Lymphocytes were removed after 1-h culture, and Mφ were obtained by further culturing monocytes for 7 days.

**Bone marrow-derived Mφ**

Annexin 1-null mice and wild-type (WT) littermate controls were bred in house (28). All animals were fed on a standard chow pellet diet with free access to water and maintained on a 12-h light-dark cycle. Animal work was performed in accordance with Home Office regulations, Animals (Scientific Procedures) Act 1986.

WT and annexin 1-null mice (4–6 wk old) were killed, and the femurs were excised. The epiphyses were removed, and bone marrow was flushed from the bones with phosphate-buffered saline (PBS) containing one or more PMN in at least five fields (minimum of 400 cells) was expressed as a percentage of the total number of Mφ and an average between duplicate wells was calculated.

For inhibitor studies, Mφ were treated with the stable cell-permeable cAMP analog 8-Br-cAMP (2 mM), the protein kinase A (PKA) inhibitor Rp-cAMP (100 μM), or the neutralizing LCPS1 Ab (1/100) in the presence or absence of LXA4 or Ac2–26, or Dex-treated Mφ for 15 min at 37°C before coincubation with apoptotic PMNs as described above.

**Actin staining**

Mφ on glass coverslips were incubated with vehicle or Ac2–26 peptide (32 μM) or supernatants from Dex-treated Mφ for 15 min at 37°C. In inhibitor studies, the neutralizing LCPS1 Ab (1/100) was coincubated with supernatants from Dex-treated Mφ. At the end of the incubations, cells were rinsed with PBS and fixed in 3.8% paraformaldehyde-PBS for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained with Oregon Green-phalloidin (0.33 μM/mL) for 30 min at room temperature. Coverslips were mounted on microscope glass slides with Probing Antifade medium (Molecular Probes). Cells were viewed on an Axiosvert 200M fluorescent microscope (Zeiss) using Axiosvision image analysis software (Imaging Associates).

**Western blotting**

Supernatants from monocytes cultured for 5 days in the absence of Dex and supernatants from Dex-treated Mφ were resolved by electrophoresis on a 12% SDS polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). The membranes were incubated with blocking buffer (TBS (20 mM Tris; 137 mM NaCl) containing 0.1% Tween 20 (TBS-T) and 5% milk) before probing with a rabbit anti-annexin 1 (1/800 in TBS-T) Ab. After incubation with a HRP-conjugated anti-rabbit secondary Ab (1/2000 in TBS-T, New England Biolabs), bound Ab was visualized with an ECL detection system (Santa Cruz Biotechnology).

**Annexin depletion**

The immunodepletion was conducted using polyclonal rabbit anti-annexin 1 (Cambridge Biosciences). Supernatants from Dex-treated Mφ were incubated overnight at 4°C with the primary Ab directed against annexin 1 or rabbit IgG (control), followed by protein G-agarose beads (Santa Cruz Biotechnology) for 2 h. The Ag-Ab immune complexes were separated by centrifugation. The supernatants were collected, and immunoprecipitation was conducted two more times. Complete annexin 1 depletion was verified by Western blotting as described above.

**Cytokine production**

TGF-β1 in acid-activated samples and IL-8 were assayed in supernatants from cocultures of Mφ and apoptotic PMNs by ELISA according to the manufacturer’s instruction. Supernatants from Mφ stimulated with 10 ng/ml LPS and coincubated with apoptotic PMNs for 18 h were used as positive controls.

**Statistical analysis**

Statistical analysis was conducted using one-way ANOVA or Student’s t test with p < 0.05 for n independent samples being deemed statistically significant.

**Results**

**Peptide Ac2–26 stimulates Mφ phagocytosis of apoptotic PMNs coupled to rearrangement of actin cytoskeleton**

Human monocyte-derived Mφ were exposed to the peptide Ac2–26 (32 μM; 15 min at 37°C) before addition of apoptotic human PMNs, and the extent of the ensuing phagocytosis was compared with that obtained with LXA4 previously shown to significantly enhance phagocytosis (7, 8). Pretreatment of Mφ with peptide Ac2–26 resulted in a significant increase of phagocytosis of apoptotic PMNs, with an effect comparable to that observed with LXA4 (1 nM; 15 min at 37°C) (control, 12.6 ± 1.5; LXA4, 21.8 ± 2.6%; Ac2–26, 20.7 ± 2.8%; p < 0.01 vs control; Fig. 1A). In addition, stimulation of Mφ with the peptide Ac2–26 caused F-actin rearrangement and modification in cell shape leading to cell polarization (Fig. 1B), suggesting that the peptide Ac2–26, similarly to the described effect of LXS, primed Mφ to a phagocytic state (3, 9).

**Supernatants from Dex-treated Mφ contain annexin 1 and are associated with enhanced phagocytosis and actin rearrangement**

As discussed above, annexin 1 protein is up-regulated by glucocorticoids to mediate some of their anti-inflammatory properties (15–21). We investigated the potential role of endogenous annexin 1 on phagocytosis of apoptotic cells by Mφ. Human monocytes were
cultured for 5 days in the presence or absence of 1 μM Dex. Cell-free supernatants were collected and used to stimulate phagocytosis of apoptotic PMNs by Mφ.

Treatment of Mφ with supernatants from Dex-treated Mφ significantly enhanced phagocytosis of apoptotic PMNs compared with appropriate controls (vehicle; medium plus Dex incubated for 5 days without cells; supernatants from monocytes cultured for 5 days in the absence of Dex; Fig. 2A). Serial dilutions of the supernatants from Dex-treated Mφ showed that the effect on phagocytosis was linear with almost no changes in phagocytosis being observed with a 1/10 dilution (Fig. 2B). Fig. 2C shows that, similarly to the peptide Ac2–26, supernatants from Dex-treated Mφ induces actin rearrangement in Mφ, an effect reversed by coincubation with a neutralizing polyclonal Ab (LCPS1 diluted 1/100), that previously has been shown to neutralize the actions of Dex and annexin 1 (27).

The presence of annexin 1 in the supernatants from Dex-treated Mφ was assessed by Western blotting. Fig. 3A shows the presence of the characteristic annexin 1 doublet with the 34- and 37-kDa isoforms in the supernatants from Dex-treated Mφ. Annexin 1 was also detected in the supernatants from Mφ cultured for 5 days in the absence of Dex (Fig. 3A), but in lesser amounts and not in sufficient concentration to increase phagocytosis of apoptotic PMNs. The supernatants of Dex-treated Mφ were depleted of annexin 1 by three subsequent immunoprecipitations. Annexin 1-depleted supernatants failed to increase phagocytosis of apoptotic PMN by Mφ, indicating that annexin is responsible for the mechanism of action of the supernatant from Dex-treated Mφ (Fig. 3B).

To further investigate the possibility that endogenous annexin 1 could mediate the stimulatory effect produced by the supernatants...
from Dex-treated Mφ, a neutralizing polyclonal Ab that has previously shown to neutralize the actions of Dex and annexin 1 (27) was used (LCPS1 diluted 1/100). LCPS1 slightly but not significantly attenuated the phagocytosis in basal condition (16% inhibition). However, the neutralizing Ab LCPS1 significantly inhibited the effect of Ac2–26 peptide as well as Dex-treated Mφ-stimulated phagocytosis of apoptotic PMNs by 41 and 49%, respectively (Fig. 3C).

Phagocytosis of apoptotic PMNs induced by Ac2–26 peptide and by supernatants from Dex-treated Mφ is coupled to TGF-β release with a concomitant decrease in IL-8 production

Phagocytosis of apoptotic cells may contribute to the resolution of inflammation being coupled with TGF-β release and not associated with release of proinflammatory cytokines such as IL-8 and MCP-1 (4, 5, 7, 8). To investigate whether annexin-mediated phagocytosis might promote the resolution of inflammation, unstimulated Mφ (control) or treated with peptide Ac2–26 or supernatants from Dex-treated Mφ (15 min at 37°C) were exposed to annexin-depleted supernatant for 15 min before coincubation with apoptotic PMNs for 30 min. Data are expressed as percentage of phagocytosis ± SEM (n = 3). C, Mφ were treated with anti-annexin 1 Ab (LCPS1 diluted 1/100) in the absence (control) or in the presence of supernatants from Dex-treated Mφ or Ac2–26 peptide (15 min at 37°C) and then coincubated with apoptotic PMNs for 30 min. Data are expressed as fold of induction over control; mean ± SEM (n = 3); *, p < 0.05 vs absence of LCPS1.

FIGURE 3. Supernatants from Dex-treated Mφ contain annexin 1. A, Supernatants from Mφ treated with Dex (1 μM) and supernatants from monocytes cultured for 5 days in the absence of Dex were resolved by electrophoresis on a 12% SDS-polyacrylamide gel, and Western blot analysis was performed with an Ab against annexin 1. A representative blot of n = 3 is shown. B, Supernatants from Dex-treated Mφ were depleted of annexin by immunoprecipitation with an Ab direct against annexin 1. Complete annexin 1 depletion was verified by Western blotting and showed in the inset. Mφ were exposed to annexin-depleted supernatant for 15 min before coincubation with apoptotic PMNs for 30 min. Data are expressed as percentage of phagocytosis ± SEM (n = 3). Complete annexin 1 depletion was verified by Western blotting and showed in the inset. Mφ were exposed to annexin-depleted supernatant for 15 min before coincubation with apoptotic PMNs for 30 min. Data are expressed as percentage of phagocytosis ± SEM (n = 3). C, Mφ were treated with anti-annexin 1 Ab (LCPS1 diluted 1/100) in the absence (control) or in the presence of supernatants from Dex-treated Mφ or Ac2–26 peptide (15 min at 37°C) and then coincubated with apoptotic PMNs for 30 min. Data are expressed as fold of induction over control; mean ± SEM (n = 3); *, p < 0.05 vs absence of LCPS1.

Annexin-stimulated phagocytosis of apoptotic PMNs is blocked by the FPR antagonist Boc1 and is cAMP/PKA sensitive

It has recently been demonstrated that full-length annexin 1 and its bioactive peptides interact with the LX receptor ALXR/FPRL1 (12, 13, 29). In our experimental conditions, the effect of Ac2–26 (32 μM) on the uptake of apoptotic PMNs was not additive with a suboptimal (10–12 M) ineffective concentration of LXA4 (vehicle, 11.5 ± 1.7; LXA4, 13.08 ± 1.9; Ac2–26, 18.6 ± 1.7; LXA4 plus Ac2–26, 14.6 ± 1.1; percentage of phagocytosis ± SEM; n = 3; *, p < 0.05 vs vehicle). These data suggest that the annexin effect on phagocytosis could be mediated by the ALXR/FPRL1 receptor. To address this possibility further, we used the Boc-1 compound, an antagonist to this receptor previously shown to antagonize the effects of the peptide Ac2–26 on human neutrophils (29, 30). In our experimental conditions, the stimulating effect of either peptide Ac2–26 or endogenous annexin 1 (present in the supernatants from Dex-treated Mφ) on phagocytosis of apoptotic PMNs was significantly inhibited by Boc1 (Fig. 5). The antagonist was equally effective on the response elicited by LXA4. Together, these data indicate a relevant function for the ALXR/FPRL1 receptor in this specific Mφ response.

The cAMP/PKA pathway is one of the signal transduction pathways better characterized in the phagocytosis of apoptotic PMNs (31). Consistent with our previous results for LXs (7), in this study,
we demonstrate that elevation of cAMP levels with a cell-permeant analog of cAMP, the 8-Br-cAMP, resulted in a significant inhibition of the effects of peptide Ac2–26 and of the Dex-treated Mφ-induced phagocytosis (Table I). On the contrary, a PKA inhibitor, the Rp-cAMP, increased phagocytosis, but no additive effect with Ac2–26 and with Dex-treated Mφ was observed, suggesting again that these compounds might be sharing intracellular pathways.

Bone marrow-derived Mφ from annexin 1-null mice show defective phagocytosis of apoptotic PMNs

To investigate the direct involvement of Mφ annexin 1 in phagocytosis of apoptotic cells, bone marrow-derived Mφ isolated from WT or annexin 1-null mice were exposed for 30 min to human apoptotic PMNs. Annexin 1-null Mφ showed a reduction of phagocytic activity (~40%) when fed with apoptotic PMNs compared with Mφ taken from littermate control mice (Fig. 6).

Discussion

There is growing appreciation that the resolution of inflammation is a dynamically regulated process that may provide several opportunities for therapeutic intervention (6). In this regard, it is noteworthy that the biosynthesis of specific mediators that actively promote the resolution of inflammation has been demonstrated (10, 11, 32). An important component of effective host defense requires leukocyte trafficking to a site of inflammation where they perform their specialized roles. Subsequent to apoptosis, it is vital that effete leukocytes are cleared by nonphlogistic phagocytosis, sparing tissue from exposure to the noxious and immunogenic contents of necrotic cells. Dysregulated clearance of apoptotic cells has been proposed to contribute to pathogenesis of several chronic inflammatory conditions including systemic lupus erythematosus and rheumatoid arthritis (3). Immune-suppressive glucocorticoids such as Dex are typically used in the therapeutic regimens for such conditions.

In the present study, we report the novel finding that anti-inflammatory peptide Ac2–26 (corresponding to the first amino acids of the annexin 1 N terminus) and endogenous annexin 1 released by Dex-treated Mφ promote the nonphlogistic phagocytosis of apoptotic PMNs. In addition, we demonstrate that bone marrow-derived Mφ isolated from annexin 1-null mice present a defective phagocytosis of apoptotic PMNs.

Annexin 1 was originally identified as a mediator of many of the anti-inflammatory actions of glucocorticoids (15, 16). However, it has been shown that annexin mimics the anti-inflammatory actions of glucocorticoids in many experimental models of inflammation (33), block leukocyte migration (17, 18), inhibit eicosanoid synthesis (34), and induce apoptosis (22). Autoantibodies against annexin 1 may be responsible for some forms of glucocorticoid resistance in association with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (35, 36). A role for endogenous annexin in phagocytosis of apoptotic cells has been hypothesized by the observation that annexin 1 is exported from the cytosol to the plasma membrane of apoptotic Jurkat cells where it colocalizes with PS in discrete patches (23). The exposure of annexin 1 is required for the clustering of the PS receptor, and it is necessary for an efficient recognition and internalization of apoptotic cells by endothelial cells, suggesting a role for annexin 1 as bridging molecule of PS on apoptotic cells to phagocytes (23). In addition, a role for Mφ surface expression of annexins 1 and II has recently been reported (24). Furthermore, recent work with cells taken from annexin 1-null mice indicate that lack of annexin 1 is functionally associated with a reduced capacity of the Mφ to phagocytose nonopsonized zymosan particles (37).

It was therefore of interest to us to investigate whether exogenous and endogenous annexin 1 could modulate phagocytosis of apoptotic cells and the underlying mechanisms. In the present study, we report that the peptide Ac2–26 may have a role in the resolution of inflammation by promoting the nonphlogistic phagocytosis of apoptotic PMNs. This effect is coupled to TGF-β1 release and to changes in F-actin reorganization in Mφ. Given that

### Table I. Annexin-induced phagocytosis of apoptotic PMNs by Mφ: role of cAMP and PKA

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>11.2 ± 1.5</td>
</tr>
<tr>
<td>Ac2–26</td>
<td>20.4 ± 3.3*</td>
</tr>
<tr>
<td>Supernatant from Dex-treated Mφ</td>
<td>15.4 ± 1.6*</td>
</tr>
<tr>
<td>8-Br-cAMP + vehicle</td>
<td>12.4 ± 3.4</td>
</tr>
<tr>
<td>Br-cAMP + Ac2–26</td>
<td>11.0 ± 1.0</td>
</tr>
<tr>
<td>Br-cAMP + supernatant from Dex-treated Mφ</td>
<td>10.6 ± 2.2</td>
</tr>
<tr>
<td>Rp-cAMP + vehicle</td>
<td>18.2 ± 2.3*</td>
</tr>
<tr>
<td>Rp-cAMP + Ac2–26</td>
<td>17.6 ± 1.1*</td>
</tr>
<tr>
<td>Rp-cAMP + supernatant from Dex-treated Mφ</td>
<td>19.8 ± 1.0*</td>
</tr>
</tbody>
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* Mean ± SEM (n = 4).
* p < 0.05 vs vehicle.
the process of phagocytosis is highly dependent on the localized polymerization of actin filaments that facilitate the formation of filopodia that surround the cells or the microorganisms to be engulfed (38), it can be suggested that the effect of Ac2–26, on actin cytoskeleton might prime Mφ to a phagocytic phenotype, contributing to the potential role of peptide Ac2–26 in promoting phagocytosis.

Resting Mφ contain high levels of annexin 1 (39), which is rapidly externalized by glucocorticoid treatment (39–41). Similarly, the monocytic cell line U937 expresses increased amount of endogenous annexin 1 during differentiation by phorbol ester, and subsequent glucocorticoid treatment results in translocation of annexin 1 to the external surface and its release into the medium (42, 43). In this study, we present evidence that the endogenous annexin released by Dex-treated Mφ displays a proinflammatory effect of apoptotic cells. The effect of supernatants from Dex-treated Mφ was mediated specifically by annexin and not by Dex itself present in the medium, because the controls prepared with medium containing serum and Dex failed to increase phagocytosis. We also demonstrated that LCPS1, a neutralizing Ab raised against the N-terminal peptide of annexin 1 (27), abrogated the enhancing effect of supernatant from Dex-treated Mφ. Basal phagocytosis was only slightly affected by LCPS1 Ab (−16%). Interestingly, Fan et al. (24) have demonstrated that phagocytosis of apoptotic lymphocytes by J744 cells or elicited peritoneal Mφ can be blocked by an anti-annexin 1 Ab. We have showed that immunodepletion of annexin 1 from the supernatants of Dex-treated Mφ by immunoprecipitation abolished the stimulating effect on phagocytosis. For immunodepletion, we use a rabbit polyclonal Ab that recognized the full-length annexin 1 and that is well characterized for Western blotting according to the manufacturer’s details, but not for neutralizing effect. On the contrary, the LCPS1 Ab is a polyclonal sheep serum raised against the annexin 1-derived peptide Ac2–26 that neutralizes the action of Dex and annexin 1 (27). In aggregate, these data support the hypothesis that endogenous annexin is the mediator responsible for the proinflammatory effect, suggesting that Mφ-derived annexin 1 could be one of the mechanisms responsible for glucocorticoid facilitation of the uptake of apoptotic PMNs, eosinophils, and lymphocytes (25, 26). We cannot exclude the possibility that LCPS1 neutralizes Ac2–26 peptide generated by proteolysis of annexin 1 in the supernatants of Dex-treated Mφ and that Ac2–26 peptide is responsible for the stimulation of phagocytosis. However, we have now shown that the annexin 1 mimetics (shorter peptide sequences from the N-terminal region of the protein, i.e., Ac2–26) share some of the anti-inflammatory action of annexin 1 (12, 16, 19, 29). Hannon et al. (28) have recently generated a mouse line deficient in annexin 1. These mice are more prone to inflammatory responses in models of acute (28, 44) and chronic (45) inflammation. In addition, peritoneal Mφ deficient in annexin 1 display a stimulus-dependent alteration in phagocytosis of insoluble stimuli; for instance, defect in the ingestion of non-opsonized, but not of opsonized, zymosan was evident (37). In this study, we have used bone marrow Mφ in a heterologous system with human PMNs, as previously described (8, 25, 46, 47), to determine the role for endogenous annexin 1. In line with the neutralizing experiments, bone marrow-derived Mφ from annexin 1-null mice had a reduced phagocytic activity indicating a physiological role of endogenous annexin 1. These data are consistent with a role for endogenous Mφ annexin in facilitating phagocytosis of apoptotic leukocytes as described by Fan et al. (24). We can therefore propose that at least part of the anti-inflammatory effects lost by Dex, and possibly other glucocorticoids, when the later stages of the inflammatory responses are investigated in annexin 1-null mice (44, 45), could be due to a defective removal of apoptotic cells, hence to a malfunctioning healing process.

Recent investigations conducted predominantly with human neutrophils have indicated an involvement of the receptor for formylated peptides, termed FPR, and its analog FPRL1 (or ALXR, because it is the receptor for the endogenous ligand LXA4) in the in vitro inhibitory actions of annexin 1 and its peptidomimetics (13, 29). LXA4 and annexin 1 and its bioactive peptides converge on ALXR to down-regulate PMN recruitment to inflammatory loci (13). Results in the same study suggested that the peptide Ac2–26 and LXA4 can share some intracellular signaling pathways in PMNs (13). In the mouse, the antiadhesive and anti-inflammatory actions of peptide Ac2–26 on PMNs are mediated by a Boc-1-sensitive receptor, distinct from FPR (12). In the present paper, we report that LXA4 and annexin-stimulated phagocytosis of apoptotic PMNs was inhibited by a pan-FPR/ALXR antagonist, Boc1, suggesting the involvement of a common receptor mechanism. Annexin 1 colocalizes with PS on the cell membrane of apoptotic cells (23), and it has been hypothesized that, when annexin 1 (or possibly annexin II) is bound to PS on the target cells, it can potentially bind to annexin receptor on the Mφ surface or function as bridging molecule (24). We cannot rule out the possibility that annexin acts as a bridging molecule; however, our findings with Boc1 and the lack of additivity of the effect of peptide Ac2–26 and LXA4 in stimulating phagocytosis suggest a predominant role for ALXR in the uptake of apoptotic PMNs. The ALXR is a pertussis toxin-sensitive G protein-coupled receptor (48, 49).

We have previously shown that LX-stimulated phagocytosis and actin cytoskeleton rearrangement involves cAMP/PKA pathways (7, 9), one of the intracellular signaling pathways that controls uptake of apoptotic cells (31). In this study, we report that both peptide Ac2–26 and Dex-treated Mφ-stimulated phagocytosis was inhibited by cell-permeable 8-Br-cAMP and mimicked by a PKA inhibitor, suggesting a modulatory role for intracellular cAMP also in the annexin-stimulated phagocytosis.

In conclusion, we report for the first time that a peptidomimetic of annexin 1 promotes phagocytosis of apoptotic cells acting through the ALXR receptor and stimulating F-actin reorganization in Mφ. Such an effect could be translated to the endogenous protein, because this proinflammatory effect was observed when endogenous annexin was released by Dex-treated Mφ and was markedly attenuated in cells prepared from annexin 1-null mice. Altogether, these novel actions expand and strengthen the role for annexin 1 as a proresolving mediator of the inflammatory milieu.

Acknowledgment
We thank Dr. Cormac Taylor for helpful discussion.

Disclosures
The authors have no financial conflict of interest.

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