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# Modulation of Phagocytosis of Apoptotic Neutrophils by Supernatant from Dexamethasone-Treated Macrophages and Annexin-Derived Peptide Ac<sub>2-26</sub><sup>1</sup>

Paola Maderna,<sup>2\*</sup> Simon Yona,<sup>†</sup> Mauro Perretti,<sup>†</sup> 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 (Ac<sub>2-26</sub>) significantly stimulates nonphlogistic phagocytosis of apoptotic polymorphonuclear leukocytes (PMNs) by human monocyte-derived macrophages (M $\phi$ ). Peptide Ac<sub>2-26</sub>-stimulated phagocytosis is accompanied by rearrangement of the M $\phi$  actin cytoskeleton. To investigate the potential role of endogenous annexin on clearance of apoptotic cells, M $\phi$  were cultured for 5 days in the presence of dexamethasone. Supernatants collected from dexamethasone-treated M $\phi$  significantly enhanced the ability of naive M $\phi$  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 M $\phi$  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 (M $\phi$ )<sup>3</sup> 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- $\beta$ <sub>1</sub> and PGE<sub>2</sub> and the suppression of release of proinflammatory mediators (4, 5). In this context, endogenous and exogenous regulators of this process may have therapeutical potential (6). We have previously demonstrated that endogenously produced lipoxin (LX), LXA<sub>4</sub> and LXB<sub>4</sub>, and stable LX synthetic analogs can promote nonphlogistic phagocytosis of apoptotic polymorphonuclear

leukocytes (PMNs) by human monocyte-derived M $\phi$  in vitro (7) and in vivo (8). This prophagocytic effect seems to be coupled to LX-mediated alterations of the M $\phi$  actin cytoskeleton (9) and further expands on the potential proresolution bioactions of LXs (10, 11).

In this study, we have investigated whether a peptide mimetic of annexin 1, Ac<sub>2-26</sub>, a recently described agonist for the LXA<sub>4</sub> 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 M $\phi$  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 Ac<sub>2-26</sub> promotes phagocytosis of apoptotic PMNs by human M $\phi$ . 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 M $\phi$  from annexin 1-null mice are defective in clearance of apoptotic cells.

## Materials and Methods

### Materials

LXA<sub>4</sub> was obtained from Biomol Research Laboratories. The annexin 1 mimetic peptide Ac<sub>2-26</sub> (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) was prepared by the Advance Biotechnology Centre (The Charing Cross and Westminster Medical School, London, U.K.) by using solid-phase

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<sup>3</sup> Abbreviations used in this paper: M $\phi$ , macrophage; LX, lipoxin; PMN, polymorphonuclear leukocyte; ALXR, LXA<sub>4</sub> receptor; PS, phosphatidylserine; Dex, dexamethasone; Rp-cAMP, adenosine 3',5'-cyclic monophosphorothioate; 8-Br-cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; FPR, formyl-peptide receptor; WT, wild type; PKA, protein kinase A.

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 Ac<sub>2-26</sub> (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-Aldrich. The antagonist Boc1 (*N*-*t*-Boc-Phe-D-Leu-Phe-D-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- $\beta_1$  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,  $\leq 0.1\%$ ). 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  $2 \times 10^6$  cells/ml in RPMI 1640 (BioWhittaker) supplemented with 10% autologous serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin (Invitrogen Life Technologies). Lymphocytes were removed after 1-h culture, and M $\phi$  were obtained by further culturing monocytes for 7 days.

For preparation of supernatant from Dex-treated M $\phi$ , monocytes prepared as above were cultured for 5 days in RPMI 1640 supplemented with 10% serum in the presence or absence of 1  $\mu$ M Dex. The supernatants were collected, centrifuged at 14,000 rpm for 10 min, and stored at  $-20^\circ\text{C}$ .

PMNs, after dextran sedimentation (Dextran T500; Pharmacia) and hypotonic lysis of red cells, were suspended at  $4 \times 10^6$  cells/ml. Spontaneous apoptosis was achieved by culturing PMNs in RPMI 1640 supplemented with 10% autologous serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin for 20 h at  $37^\circ\text{C}$  in 5% CO<sub>2</sub> atmosphere. Cells were on average 25–50% apoptotic with <3% necrosis as assessed by light microscopy on stained cytocentrifuged preparations.

### Bone marrow-derived M $\phi$

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 bone before the marrow plugs being mechanically disrupted and washed ( $400 \times g$ ; 10 min). Cells were then resuspended in RPMI 1640 supplemented with 25 mM HEPES, 10% FCS, and 20% L929 conditioned medium, and incubated on plastic at  $37^\circ\text{C}$ . Fresh culture medium was added on day 3 and at day 6. Cultures were confluent by day 7. Cultures were incubated with PBS supplemented with 10 mM EDTA and 4 mg/ml lidocaine-HCl for 10 min before removal for cell harvesting and seeded on 24-well plates at  $1 \times 10^6$  cells/well.

### M $\phi$ phagocytosis of apoptotic PMNs

Human M $\phi$  were treated with the appropriate stimuli as indicated for 15 min at  $37^\circ\text{C}$ . The treated cells were washed with RPMI 1640 before cocultivation with apoptotic PMNs ( $4 \times 10^6$  PMNs/well) at  $37^\circ\text{C}$  for 30 min. Mouse bone marrow-derived M $\phi$  were exposed to human apoptotic PMNs at  $37^\circ\text{C}$  for 30 min. Noingested cells were removed by three washes with cold PBS. Phagocytosis was assayed by myeloperoxidase staining of cocultures fixed with 2.5% glutaraldehyde as previously reported (7). For each experiment, the number of M $\phi$  containing one or more PMN in at least five fields (minimum of 400 cells) was expressed as a percentage of the total number of M $\phi$  and an average between duplicate wells was calculated.

For inhibitor studies, M $\phi$  were treated with the stable cell-permeable cAMP analog 8-Br-cAMP (2 mM), the protein kinase A (PKA) inhibitor Rp-cAMP (100  $\mu$ M), Boc1 (100  $\mu$ M), or the neutralizing LCPS1 Ab (1:100) in the presence or absence of LXA<sub>4</sub> or Ac<sub>2-26</sub> or Dex-treated M $\phi$  for 15 min at  $37^\circ\text{C}$  before cocultivation with apoptotic PMNs as described above.

### Actin staining

M $\phi$  on glass coverslips were incubated with vehicle or Ac<sub>2-26</sub> peptide (32  $\mu$ M) or supernatants from Dex-treated M $\phi$  for 15 min at  $37^\circ\text{C}$ . In inhibitory studies, the neutralizing LCPS1 Ab (1:100) was cocultured with supernatants from Dex-treated M $\phi$ . 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  $\mu$ mol/L) for 30 min at room temperature. Coverslips were mounted on microscope glass slides with Probing Antifade medium (Molecular Probes). Cells were viewed on an Axiovert 200M fluorescent microscope (Zeiss) using Axiovision image analysis software (Imaging Associates).

### Western blotting

Supernatants from monocytes cultured for 5 days in the absence of Dex and supernatants from Dex-treated M $\phi$  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 mmol/L Tris; 137 mmol/L NaCl) containing 0.1% Tween 20 (TBS-T) and 5% milk) before probing with a rabbit anti-annexin 1 (1/500 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 M $\phi$  were incubated overnight at  $4^\circ\text{C}$  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- $\beta_1$  in acid-activated samples and IL-8 were assayed in supernatants from cocultures of M $\phi$  and apoptotic PMNs by ELISA according to the manufacturer's instruction. Supernatants from M $\phi$  stimulated with 10 ng/ml LPS and cocultured with apoptotic PMNs for 18 h were used as positive controls.

### Statistical analysis

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

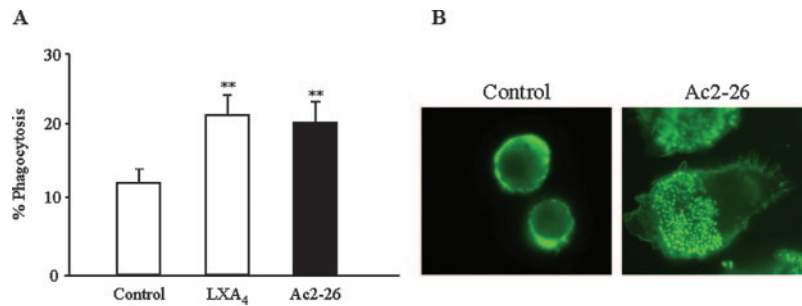
## Results

### Peptide Ac<sub>2-26</sub> stimulates M $\phi$ phagocytosis of apoptotic PMNs coupled to rearrangement of actin cytoskeleton

Human monocyte-derived M $\phi$  were exposed to the peptide Ac<sub>2-26</sub> (32  $\mu$ M; 15 min at  $37^\circ\text{C}$ ) before addition of apoptotic human PMNs, and the extent of the ensuing phagocytosis was compared with that obtained with LXA<sub>4</sub>, previously shown to significantly enhance phagocytosis (7, 8). Pretreatment of M $\phi$  with peptide Ac<sub>2-26</sub> resulted in a significant increase of phagocytosis of apoptotic PMNs, with an effect comparable to that observed with LXA<sub>4</sub> (1 nM; 15 min at  $37^\circ\text{C}$ ) (control,  $12.6 \pm 1.5$ ; LXA<sub>4</sub>,  $21.8 \pm 2.6^{**}$ ; Ac<sub>2-26</sub>,  $20.7 \pm 2.8^{**}$ ; % phagocytosis  $\pm$  SEM;  $n = 12$ ; \*\*,  $p < 0.01$  vs control; Fig. 1A). In addition, stimulation of M $\phi$  with the peptide Ac<sub>2-26</sub> caused F-actin rearrangement and modification in cell shape leading to cell polarization (Fig. 1B), suggesting that the peptide Ac<sub>2-26</sub>, similarly to the described effect of LXs, primed M $\phi$  to a prophagocytic state (3, 9).

### Supernatants from Dex-treated M $\phi$ 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 M $\phi$ . Human monocytes were



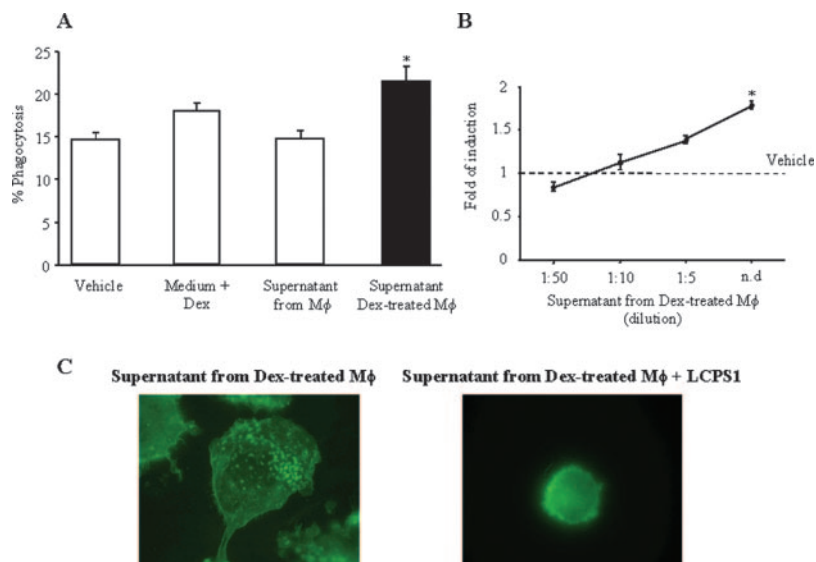
**FIGURE 1.** Peptide Ac<sub>2-26</sub> stimulates phagocytosis of apoptotic PMNs by M $\phi$  and actin reorganization in M $\phi$ . *A*, M $\phi$  were treated with vehicle (control), LXA<sub>4</sub> (1 nM), or peptide Ac<sub>2-26</sub> (32  $\mu$ M) for 15 min before coincubation with apoptotic PMNs for 30 min. Phagocytosis was quantified as described in *Materials and Methods*. Data are expressed as percentage of phagocytosis  $\pm$  SEM ( $n = 12$ ); \*\*,  $p < 0.01$  vs control. *B*, M $\phi$  were exposed to vehicle (control) or peptide Ac<sub>2-26</sub> (32  $\mu$ M) for 15 min at 37°C. Cells were fixed with paraformaldehyde, and localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a  $\times 100$  oil objective. Images are representative of one of three independent experiments.

cultured for 5 days in the presence or absence of 1  $\mu$ M Dex. Cell-free supernatants were collected and used to stimulate phagocytosis of apoptotic PMNs by M $\phi$ .

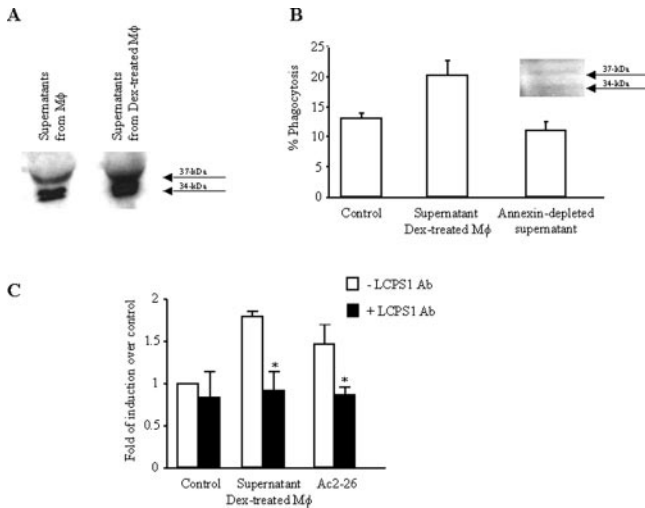
Treatment of M $\phi$  with supernatants from Dex-treated M $\phi$  significantly enhanced phagocytosis of apoptotic PMNs compared with appropriate controls (vehicle; medium plus Dex incubated for 5 days without cells; supernatants from monocytes cultured for 5 days in the absence of Dex; Fig. 2*A*). Serial dilutions of the supernatants from Dex-treated M $\phi$  showed that the effect on phagocytosis was linear with almost no changes in phagocytosis being observed with a 1/10 dilution (Fig. 2*B*). Fig. 2*C* shows that, similarly to the peptide Ac<sub>2-26</sub>, supernatants from Dex-treated M $\phi$  induces actin rearrangement in M $\phi$ , 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 M $\phi$  was assessed by Western blotting. Fig. 3*A* shows the presence of the characteristic annexin 1 doublet with the 34- and 37-kDa isoforms in the supernatants from Dex-treated M $\phi$ . Annexin 1 was also detected in the supernatants from M $\phi$  cultured for 5 days in the absence of Dex (Fig. 3*A*), but in lesser amounts and not in sufficient concentration to increase phagocytosis of apoptotic PMNs. The supernatants of Dex-treated M $\phi$  were depleted of annexin 1 by three subsequent immunoprecipitations. Annexin 1-depleted supernatants failed to increase phagocytosis of apoptotic PMN by M $\phi$ , indicating that annexin is responsible for the mechanism of action of the supernatant from Dex-treated M $\phi$  (Fig. 3*B*). The efficacy of our immunodepletion procedure was investigated by Western blotting (Fig. 3*B*, *inset*).

To further investigate the possibility that endogenous annexin 1 could mediate the stimulatory effect produced by the supernatants



**FIGURE 2.** Supernatants from Dex-treated M $\phi$  promote phagocytosis of apoptotic PMNs by M $\phi$ . *A*, Human monocytes were cultured for 5 days with medium RPMI 1640 plus 10% autologous serum in the absence or presence of 1  $\mu$ M Dex. Supernatants were collected, centrifuged, and stored. Human M $\phi$  derived from adherent monocytes cultured for 7 days were exposed to the supernatants from Dex-treated M $\phi$  or appropriate control (vehicle, medium plus Dex incubated for 5 days without cells or supernatants from monocytes cultured for 5 days in the absence of Dex) and incubated for 15 min before coincubation with apoptotic PMNs for 30 min. Data are expressed as percentage of phagocytosis  $\pm$  SEM ( $n = 10$ ); \*,  $p < 0.01$ . *B*, Supernatants from Dex-treated M $\phi$  were serially diluted with RPMI 1640 medium and used as stimuli for phagocytosis of apoptotic PMNs as described above. Data are expressed as fold of induction over vehicle (mean  $\pm$  SEM;  $n = 4$ ; \*,  $p < 0.05$ ). *C*, M $\phi$  were exposed to supernatants from Dex-treated M $\phi$  in the absence or in the presence of LCPS1 Ab for 15 min at 37°C. Cells were fixed with paraformaldehyde, and localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a  $\times 100$  oil objective. Images are representative of one of three independent experiments.

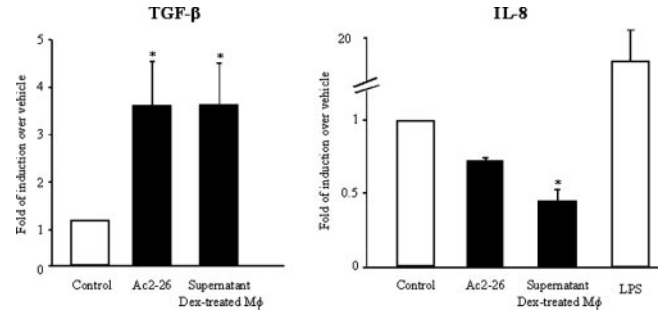


**FIGURE 3.** Supernatants from Dex-treated Mφ contain annexin 1. *A*, Supernatants from Mφ treated with Dex (1 μM) and supernatants from monocytes cultured for 5 days in the absence of Dex were resolved by electrophoresis on a 12% SDS-polyacrylamide gel, and Western blot analysis was performed with 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  $\pm$  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 Ac<sub>2-26</sub> 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  $\pm$  SEM ( $n = 3$ ); \*,  $p < 0.05$  vs absence of LCPS1.

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 Ac<sub>2-26</sub> 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 Ac<sub>2-26</sub> 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<sub>1</sub> 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 Ac<sub>2-26</sub> or supernatants from Dex-treated Mφ (15 min) were exposed to apoptotic PMNs, and the cytokine levels were quantified in the supernatants by ELISA. As previously described (4), in our experimental conditions, levels of TGF-β<sub>1</sub> produced by unstimulated Mφ in the absence of apoptotic cells were low (data not shown). Ac<sub>2-26</sub> peptide or supernatants from Dex-treated Mφ treatment induced a further significant increase in TGF-β<sub>1</sub> levels after 30-min coincubation with apoptotic PMNs compared with control (Fig. 4). In contrast, Ac<sub>2-26</sub> peptide or supernatants from Dex-treated Mφ treatment caused a further slight decrease in IL-8 production compared with unstimulated Mφ that had ingested apoptotic PMNs



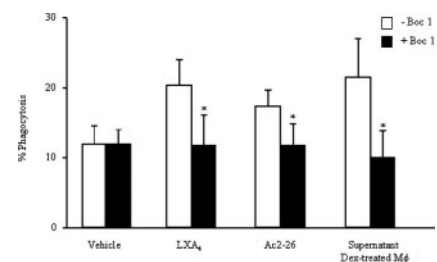
**FIGURE 4.** Phagocytosis of apoptotic PMNs induced by Ac<sub>2-26</sub> peptide and by supernatants from Dex-treated Mφ is nonphlogistic. Mφ were unstimulated (control) or treated with Ac<sub>2-26</sub> (32 μM) or supernatants from Dex-treated Mφ for 15 min at 37°C before coincubation with apoptotic PMNs. After 30 min, supernatants were collected and TGF-β<sub>1</sub> and IL-8 production was measured by ELISA. Data are expressed as fold of induction on basal value; mean  $\pm$  SEM ( $n = 4$ ). \*  $p < 0.05$  vs control.

(Fig. 4); whereas the positive control (Mφ stimulated with LPS) induced a 20-fold increase in IL-8 release.

*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 Ac<sub>2-26</sub> (32 μM) on the uptake of apoptotic PMNs was not additive with a suboptimal (10<sup>-12</sup> M) ineffective concentration of LXA<sub>4</sub> (vehicle, 11.5  $\pm$  1.7; LXA<sub>4</sub>, 13.08  $\pm$  1.9; Ac<sub>2-26</sub>, 18.6  $\pm$  1.7\*; LXA<sub>4</sub> plus Ac<sub>2-26</sub>, 14.6  $\pm$  1.1; percentage of phagocytosis  $\pm$  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 Ac<sub>2-26</sub> on human neutrophils (29, 30). In our experimental conditions, the stimulating effect of either peptide Ac<sub>2-26</sub> 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 LXA<sub>4</sub>. 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,



**FIGURE 5.** Annexin-stimulated phagocytosis of apoptotic PMNs is blocked by the FPR antagonist Boc1. Mφ were treated with Boc1 compound (100 μM) in the absence or in the presence of LXA<sub>4</sub> (1 nM) or Ac<sub>2-26</sub> peptide (32 μM) or supernatants from Dex-treated Mφ (15 min at 37°C) and then coincubated with apoptotic PMNs for 30 min. Data are mean percentage of phagocytosis  $\pm$  SEM ( $n = 3$ ). \*,  $p < 0.05$  vs -Boc1.

Table I. Annexin-induced phagocytosis of apoptotic PMNs by M $\phi$ : role of cAMP and PKA<sup>a</sup>

	% Phagocytosis
Vehicle	11.2 $\pm$ 1.5
Ac <sub>2-26</sub>	20.4 $\pm$ 3.3*
Supernatant from Dex-treated M $\phi$	15.4 $\pm$ 1.6*
8-Br-cAMP + vehicle	12.4 $\pm$ 3.4
Br-cAMP + Ac <sub>2-26</sub>	11.0 $\pm$ 1.0
Br-cAMP + supernatant from Dex-treated M $\phi$	10.6 $\pm$ 2.2
Rp-cAMP + vehicle	18.2 $\pm$ 2.3*
Rp-cAMP + Ac <sub>2-26</sub>	17.6 $\pm$ 1.1*
Rp-cAMP + supernatant from Dex-treated M $\phi$	19.8 $\pm$ 1.0*

<sup>a</sup> Mean  $\pm$  SEM ( $n = 4$ ).

\*,  $p < 0.05$  vs vehicle.

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 Ac<sub>2-26</sub> and of the Dex-treated M $\phi$ -induced phagocytosis (Table I). On the contrary, a PKA inhibitor, the Rp-cAMP, increased phagocytosis, but no additive effect with Ac<sub>2-26</sub> and with Dex-treated M $\phi$  was observed, suggesting again that these compounds might be sharing intracellular pathways.

#### Bone marrow-derived M $\phi$ from annexin 1-null mice show defective phagocytosis of apoptotic PMNs

To investigate the direct involvement of M $\phi$  annexin 1 in phagocytosis of apoptotic cells, bone marrow-derived M $\phi$  isolated from WT or annexin 1-null mice were exposed for 30 min to human apoptotic PMNs. Annexin 1-null M $\phi$  showed a reduction of phagocytic activity ( $\sim 40\%$ ) when fed with apoptotic PMNs compared with M $\phi$  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 efferete leukocytes are cleared by nonphlogistic phagocytosis, sparing

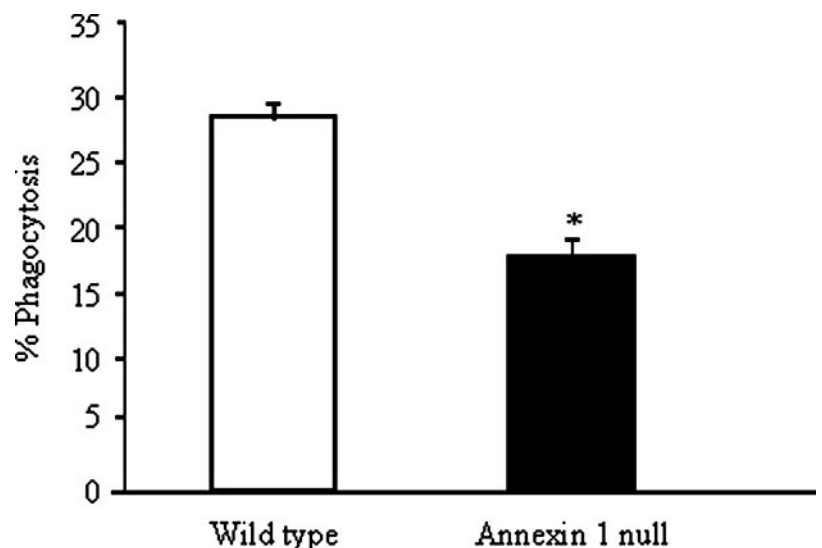
tissue from exposure to the noxious and immunogenic contents of necrotic cells. Dysregulated clearance of apoptotic cells has been proposed to contribute to pathogenesis of several chronic inflammatory conditions including systemic lupus erythematosus and rheumatoid arthritis (3). Immune-suppressive 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 Ac<sub>2-26</sub> (corresponding to the first amino acids of the annexin 1 N terminus) and endogenous annexin 1 released by Dex-treated M $\phi$  promote the nonphlogistic phagocytosis of apoptotic PMNs. In addition, we demonstrate that bone marrow-derived M $\phi$  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 M $\phi$  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 M $\phi$  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 Ac<sub>2-26</sub> may have a role in the resolution of inflammation by promoting the nonphlogistic phagocytosis of apoptotic PMNs. This effect is coupled to TGF- $\beta_1$  release and to changes in F-actin reorganization in M $\phi$ . Given that

**FIGURE 6.** Annexin 1-null M $\phi$  are defective in phagocytosis of apoptotic PMNs. Bone marrow-derived M $\phi$  from WT or annexin 1-null mice were incubated with human apoptotic PMNs for 30 min. Phagocytosis was quantified as described in *Materials and Methods*. Data are expressed as percentage of M $\phi$  staining positively for myeloperoxidase. Data are mean  $\pm$  SEM of  $n = 6$  performed with  $n = 2$  mice. \*,  $p < 0.01$  vs WT M $\phi$ .



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 Ac<sub>2-26</sub> on actin cytoskeleton might prime M $\phi$  to a phagocytotic phenotype, contributing to the potential role of peptide Ac<sub>2-26</sub> in promoting phagocytosis.

Resting M $\phi$  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 M $\phi$  displays a prophagocytic effect of apoptotic cells. The effect of supernatants from Dex-treated M $\phi$  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 M $\phi$ . 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 M $\phi$  can be blocked by an anti-annexin 1 Ab. We have showed that immunodepletion of annexin from the supernatants of Dex-treated M $\phi$  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 Ac<sub>2-26</sub> 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 prophagocytic effect, suggesting that M $\phi$ -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 Ac<sub>2-26</sub> peptide generated by proteolysis of annexin 1 in the supernatants of Dex-treated M $\phi$  and that Ac<sub>2-26</sub> peptide is responsible for the stimulation of phagocytosis. However, it has been shown that the annexin 1 mimetics (shorter peptide sequences from the N-terminal region of the protein, i.e., Ac<sub>2-26</sub>) 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 M $\phi$  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 M $\phi$  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 M $\phi$  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 M $\phi$  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 LXA<sub>4</sub>) in the in vitro inhibitory actions of annexin 1 and its peptidomimetics (13, 29). LXA<sub>4</sub> 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 Ac<sub>2-26</sub> and LXA<sub>4</sub> can share some intracellular signaling pathways in PMNs (13). In the mouse, the antiadhesive and anti-inflammatory actions of peptide Ac<sub>2-26</sub> on PMNs are mediated by a Boc-1-sensitive receptor, distinct from FPR (12). In the present paper, we report that LXA<sub>4</sub> 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 M $\phi$  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 Ac<sub>2-26</sub> and LXA<sub>4</sub> 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 Ac<sub>2-26</sub> and Dex-treated M $\phi$ -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 M $\phi$ . Such an effect could be translated to the endogenous protein, because this prophagocytic effect was observed when endogenous annexin was released by Dex-treated M $\phi$  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 proresolution mediator of the inflammatory milieu.

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## Disclosures

The authors have no financial conflict of interest.

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