The Journal of Immunology

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J Immunol 2005; 174:3727-3733; ;
doi: 10.4049/jimmunol.174.6.3727
http://www.jimmunol.org/content/174/6/3727

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Modulation of Phagocytosis of Apoptotic Neutrophils by Supernatant from Dexamethasone-Treated Macrophages and Annexin-Derived Peptide $Ac_{2–26}$[1]

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 ($Ac_{2–26}$) significantly stimulates nonphlogistic phagocytosis of apoptotic polymorphonuclear leukocytes (PMNs) by human monocyte-derived macrophages (Mϕ). Peptide $Ac_{2–26}$-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 naive 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)[3] 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_1 and PGE_2 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_4 and LXB_4, 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 proresolution bioactions of LXs (10, 11).

In this study, we have investigated whether a peptide mimetic of annexin 1, $Ac_{2–26}$, a recently described agonist for the LXA_4 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 $Ac_{2–26}$ 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

LXA_4 was obtained from Biomol Research Laboratories. The annexin 1 mimetic peptide $Ac_{2–26}$ (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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Received for publication August 20, 2004. Accepted for publication January 10, 2005.

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[1] This work was supported by the Health Research Board, Ireland (to C.G.), Enterprise Ireland (to C.G.), and The Wellcome Trust (to C.G.), and funded under the Programme for Research in Third Level Institutions, administered by the Higher Education Authority (to P.M. and C.G.). M.P. is supported by a Senior Fellowship of the Arthritis Research Campaign United Kingdom; S.Y. funding is by a Ph.D. studentship of the Nuffield Foundation, United Kingdom.

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[3] Abbreviations used in this paper: Mϕ, macrophage; LX, lipoxin; PMN, polymorphonuclear leukocyte; ALXR, LXA_4 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.

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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_{2-26} (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-Bromoadenosine 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-β_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, ≤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 2×10^6 cells/ml in RPMI 1640 (BioWhittaker) supplemented with 10% autologous serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100 μg/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. 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 μg/ml streptomycin for 20 h at 37°C in 5% CO_2 atmosphere. Cells were on average 25–50% apoptotic with <3% necrosis as assessed by light microscopy on stained cytocentrifuged preparations.

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 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°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 1×10^6 cells/well.

Mϕ phagocytosis of apoptotic PMNs

Human Mϕ were treated with the appropriate stimuli as indicated for 15 min at 37°C. The treated cells were washed with RPMI 1640 before coincubation with apoptotic PMNs (4×10^6 PMNs/well) at 37°C for 30 min. Mouse bone marrow-derived Mϕ were exposed to human apoptotic PMNs at 37°C for 30 min. Noningested 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 Mϕ 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), Boc1 (100 μM), or the neutralizing LCPS1 Ab (1:100) in the presence or absence of LXA_4 or Ac_{2-26} 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 Ac_{2-26} peptide (32 μM) or supernatants from Dex-treated Mϕ for 15 min at 37°C. In inhibitory 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 μmol/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 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 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 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-β_1 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 unpaired Student's *t* test with $p < 0.05$ for *n* independent samples being deemed statistically significant.

Results

Peptide Ac_{2-26} stimulates Mϕ phagocytosis of apoptotic PMNs coupled to rearrangement of actin cytoskeleton

Human monocyte-derived Mϕ were exposed to the peptide Ac_{2-26} (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 LXA_4, previously shown to significantly enhance phagocytosis (7, 8). Pretreatment of Mϕ with peptide Ac_{2-26} resulted in a significant increase of phagocytosis of apoptotic PMNs, with an effect comparable to that observed with LXA_4 (1 nM; 15 min at 37°C) (control, 12.6 ± 1.5; LXA_4, 21.8 ± 2.6**; Ac_{2-26}, 20.7 ± 2.8**; % phagocytosis ± SEM; $n = 12$; **, $p < 0.01$ vs control; Fig. 1*A*). In addition, stimulation of Mϕ with the peptide Ac_{2-26} caused F-actin rearrangement and modification in cell shape leading to cell polarization (Fig. 1*B*), suggesting that the peptide Ac_{2-26}, similarly to the described effect of LXs, primed Mϕ to a prophagocytic 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

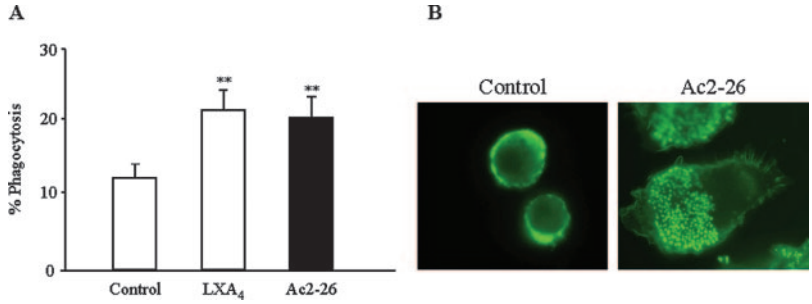

FIGURE 1. Peptide $Ac_{2–26}$ stimulates phagocytosis of apoptotic PMNs by Mϕ and actin reorganization in Mϕ. *A*, Mϕ were treated with vehicle (control), LXA_4 (1 nM), or peptide $Ac_{2–26}$ (32 μM) for 15 min before coincubation with apoptotic PMNs for 30 min. Phagocytosis was quantified as described in *Materials and Methods*. Data are expressed as percentage of phagocytosis ± SEM ($n = 12$); **, $p < 0.01$ vs control. *B*, Mϕ were exposed to vehicle (control) or peptide $Ac_{2–26}$ (32 μM) 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 ×100 oil objective. Images are representative of one of three independent experiments.

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. 2*A*). 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. 2*B*). Fig. 2*C* shows that, similarly to the peptide $Ac_{2–26}$, 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. 3*A* 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. 3*A*), 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. 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

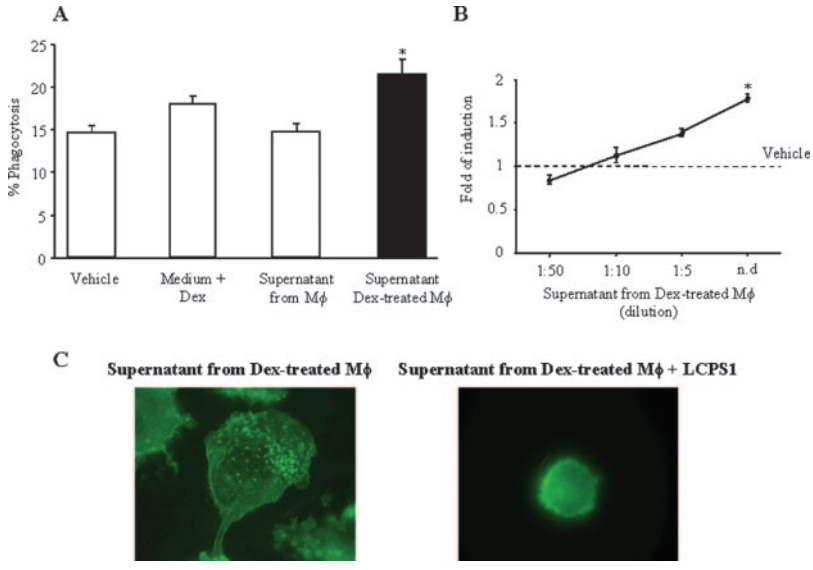

FIGURE 2. Supernatants from Dex-treated Mϕ promote phagocytosis of apoptotic PMNs by Mϕ. *A*, Human monocytes were cultured for 5 days with medium RPMI 1640 plus 10% autologous serum in the absence or presence of 1 μM Dex. Supernatants were collected, centrifuged, and stored. Human Mϕ derived from adherent monocytes cultured for 7 days were exposed to the supernatants from Dex-treated Mϕ 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 ± SEM ($n = 10$); *, $p < 0.01$. *B*, Supernatants from Dex-treated Mϕ were serial 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 ± SEM; $n = 4$; *, $p < 0.05$). *C*, Mϕ were exposed to supernatants from Dex-treated Mϕ 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 ×100 oil objective. Images are representative of one of three independent experiments.

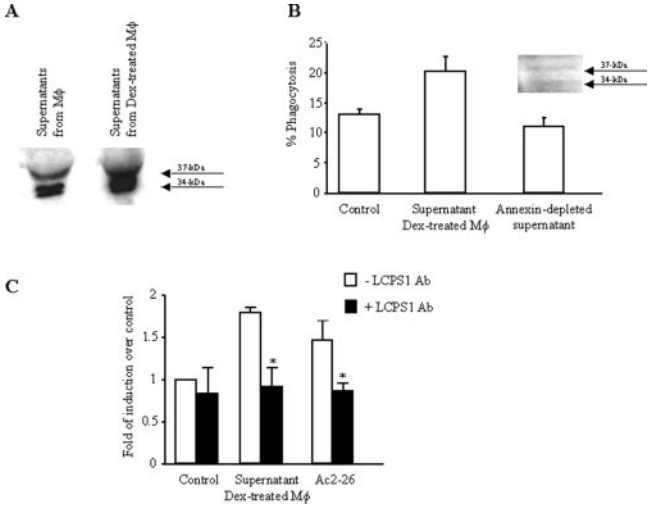

FIGURE 3. Supernatants from Dex-treated Mϕ contain annexin 1. *A*, Supernatants from Mϕ treated with Dex (1 μM) and supernatants from monocytes cultured for 5 days in the absence of Dex were resolved by electrophoresis on a 12% SDS-polyacrylamide gel, and Western blot analysis was performed with an Ab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

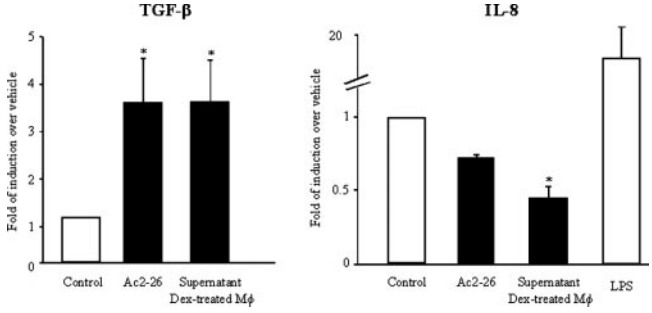

FIGURE 4. Phagocytosis of apoptotic PMNs induced by Ac_{2-26} peptide and by supernatants from Dex-treated Mϕ is nonphlogistic. Mϕ were unstimulated (control) or treated with Ac_{2-26} (32 μM) or supernatants from Dex-treated Mϕ for 15 min at 37°C before coincubation with apoptotic PMNs. After 30 min, supernatants were collected and TGF-β_1 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.

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_{2-26} peptide as well as Dex-treated Mϕ-stimulated phagocytosis of apoptotic PMNs by 41 and 49%, respectively (Fig. 3*C*).

Phagocytosis of apoptotic PMNs induced by Ac_{2-26} 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_1 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_{2-26} 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_1 produced by unstimulated Mϕ in the absence of apoptotic cells were low (data not shown). Ac_{2-26} peptide or supernatants from Dex-treated Mϕ treatment induced a further significant increase in TGF-β_1 levels after 30-min coincubation with apoptotic PMNs compared with control (Fig. 4). In contrast, Ac_{2-26} 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 (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_{2-26} (32 μM) on the uptake of apoptotic PMNs was not additive with a suboptimal (10^{-12} M) ineffective concentration of LXA_4 (vehicle, 11.5 $\pm$ 1.7; LXA_4, 13.08 $\pm$ 1.9; Ac_{2-26}, 18.6 $\pm$ 1.7*; LXA_4 plus Ac_{2-26}, 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/FPRLI 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_{2-26} on human neutrophils (29, 30). In our experimental conditions, the stimulating effect of either peptide Ac_{2-26} 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 LXA_4. 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,

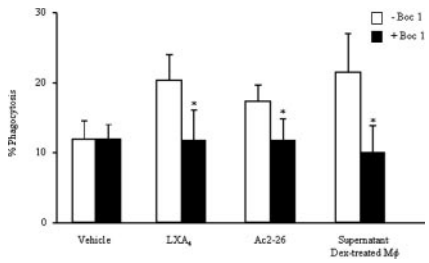

FIGURE 5. Annexin-stimulated phagocytosis of apoptotic PMNs is blocked by the FPR antagonist Boc1. Mϕ were treated with Boc1 compound (100 μM) in the absence or in the presence of LXA_4 (1 nM) or Ac_{2-26} peptide (32 μM) or supernatants from Dex-treated Mϕ (15 min at 37°C) and then coincubated with 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 Mϕ: role of cAMP and PKA*[a]

	% Phagocytosis
Vehicle	11.2 ± 1.5
$Ac_{2–26}$	20.4 ± 3.3*
Supernatant from Dex-treated Mϕ	15.4 ± 1.6*
8-Br-cAMP + vehicle	12.4 ± 3.4
Br-cAMP + $Ac_{2–26}$	11.0 ± 1.0
Br-cAMP + supernatant from Dex-treated Mϕ	10.6 ± 2.2
Rp-cAMP + vehicle	18.2 ± 2.3*
Rp-cAMP + $Ac_{2–26}$	17.6 ± 1.1*
Rp-cAMP + supernatant from Dex-treated Mϕ	19.8 ± 1.0*

[a] Mean ± 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 $Ac_{2–26}$ 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 $Ac_{2–26}$ 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 $Ac_{2–26}$ (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 $Ac_{2–26}$ may have a role in the resolution of inflammation by promoting the nonphlogistic phagocytosis of apoptotic PMNs. This effect is coupled to TGF-β_1 release and to changes in F-actin reorganization in Mϕ. Given that

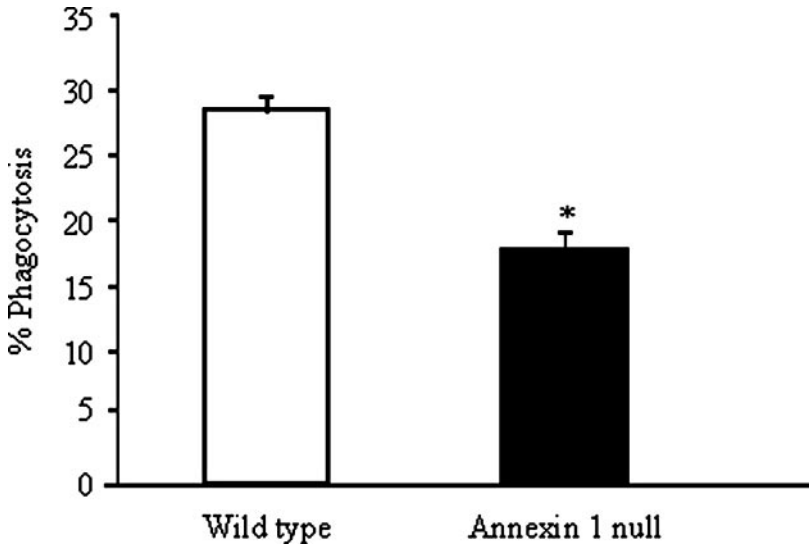

FIGURE 6. Annexin 1-null Mϕ are defective in phagocytosis of apoptotic PMNs. Bone marrow-derived Mϕ from WT or annexin 1-null mice were incubated with human apoptotic PMNs for 30 min. Phagocytosis was quantified as described in *Materials and Methods*. Data are expressed as percentage of Mϕ staining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

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_{2-26} on actin cytoskeleton might prime Mϕ to a phagocytotic phenotype, contributing to the potential role of peptide Ac_{2-26} 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 prophagocytic 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 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 Ac_{2-26} 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 prophagocytic 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 Ac_{2-26} peptide generated by proteolysis of annexin 1 in the supernatants of Dex-treated Mϕ and that Ac_{2-26} 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_{2-26}) 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 LXA_4) in the in vitro inhibitory actions of annexin 1 and its peptidomimetics (13, 29). LXA_4 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_{2-26} and LXA_4 can share some intracellular signaling pathways in PMNs (13). In the mouse, the antiadhesive and anti-inflammatory actions of peptide Ac_{2-26} on PMNs are mediated by a Boc-1-sensitive receptor, distinct from FPR (12). In the present paper, we report that LXA_4 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 Ac_{2-26} and LXA_4 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_{2-26} 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 prophagocytic 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 proresolution 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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