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CUTTING EDGE

Cutting Edge: Lipoxins Rapidly Stimulate Nonphlogistic Phagocytosis of Apoptotic Neutrophils by Monocyte-Derived Macrophages[1]

Catherine Godson,[2,3]* Siobhan Mitchell,[2]* Killeen Harvey,* Nicos A. Petasis,† Nancy Hogg,‡ and Hugh R. Brady*

Lipoxins (LX) are lipoxygenase-derived eicosanoids generated during inflammation. LX inhibit polymorphonuclear neutrophil (PMN) chemotaxis and adhesion and are putative braking signals for PMN-mediated tissue injury. In this study, we report that LXA_4 promotes another important step in the resolution phase of inflammation, namely, phagocytosis of apoptotic PMN by monocyte-derived macrophages (Mϕ). LXA_4 triggered rapid, concentration-dependent uptake of apoptotic PMN. This bioactivity was shared by stable synthetic LXA_4 analogues (picomolar concentrations) but not by other eicosanoids tested. LXA_4-triggered phagocytosis did not provoke IL-8 or monocyte chemoattractant protein-1 release. LXA_4-induced phagocytosis was attenuated by anti-CD36, $\alpha_v\beta_3$, and CD18 mAbs. LXA_4-triggered PMN uptake was inhibited by pertussis toxin and by 8-bromo-cAMP and was mimicked by Rp-cAMP, a protein kinase A inhibitor. LXA_4 attenuated PGE_2-stimulated protein kinase A activation in Mϕ. These results suggest that LXA_4 is an endogenous stimulus for PMN clearance during inflammation and provide a novel rationale for using stable synthetic analogues as anti-inflammatory compounds in vivo. ***The Journal of Immunology,*** **2000, 164: 1663–1667.**

It is increasingly appreciated that the resolution of inflammation is a dynamically regulated process. Of particular relevance in this context is the clearance of accumulated leukocytes (1, 2). Evidence from in vitro models and from histopathology suggests that neutrophil-mediated tissue damage is limited by polymorphonuclear neutrophil (PMN)[4] apoptosis and subsequent phagocytosis by macrophages (Mϕ) and other "nonprofessional" phagocytes. It is noteworthy that such phagocytic clearance is nonphlogistic; i.e., in contrast to phagocytosis of particles opsonized with complement or Ig, phagocytosis of apoptotic leukocytes does not provoke the release of proinflammatory mediators (reviewed in Refs. 1 and 2). These observations suggest the existence of a specialized phagocytic process for removal of apoptotic PMN from an inflammatory milieu. Several aspects of phagocyte-apoptotic cell recognition systems have been described involving the concerted action of cell surface molecules (reviewed in Refs. 1–3). To date, rapid modulation of PMN phagocytosis has not been described (4, 5).

Lipoxins (lipoxygenase interaction products; LX) are lipid-derived mediators typically generated by transcellular lipoxygenation of arachidonic acid. Several lines of evidence suggest that LX are braking signals for PMN recruitment in host defense, inflammation, and hypersensitivity reactions (reviewed in Ref. 6). LX have been detected in tissues and inflammatory exudates in experimental and human diseases. LX, at nanomolar concentrations, inhibit PMN chemotaxis, β_2 integrin and P-selectin-dependent PMN adhesion to endothelial cells, and PMN transmigration across confluent monolayers of endothelial and epithelial cells in vitro in response to leukotrienes and other inflammatory mediators (6–8). LX inhibit several other proinflammatory responses of leukocytes and parenchymal cells, including PMN degranulation and cytokine release by colonic epithelial cells (9, 10). Furthermore, ex vivo treatment of PMN with LXA_4 blunts their subsequent recruitment to inflamed renal glomeruli in experimental immune complex glomerulonephritis, and impaired LXA_4 biosynthesis has been associated with exaggerated PMN recruitment in the latter setting (11). Interestingly, the topical and systemic administration of LX and/or synthetic LX analogues inhibits PMN recruitment and plasma exudation induced by leukotriene B_4 and other insults in various models of acute inflammation (12, 13). Aspirin promotes the generation of LX epimers during leukocyte interactions with endothelial and epithelial cells which may account for some of the efficacy

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[4] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; Mϕ, macrophage; LX, lipoxin; PKA, protein kinase A; MPO, myeloperoxidase; MCP-1. monocyte chemoattractant protein-1; PTX, pertussis toxin.

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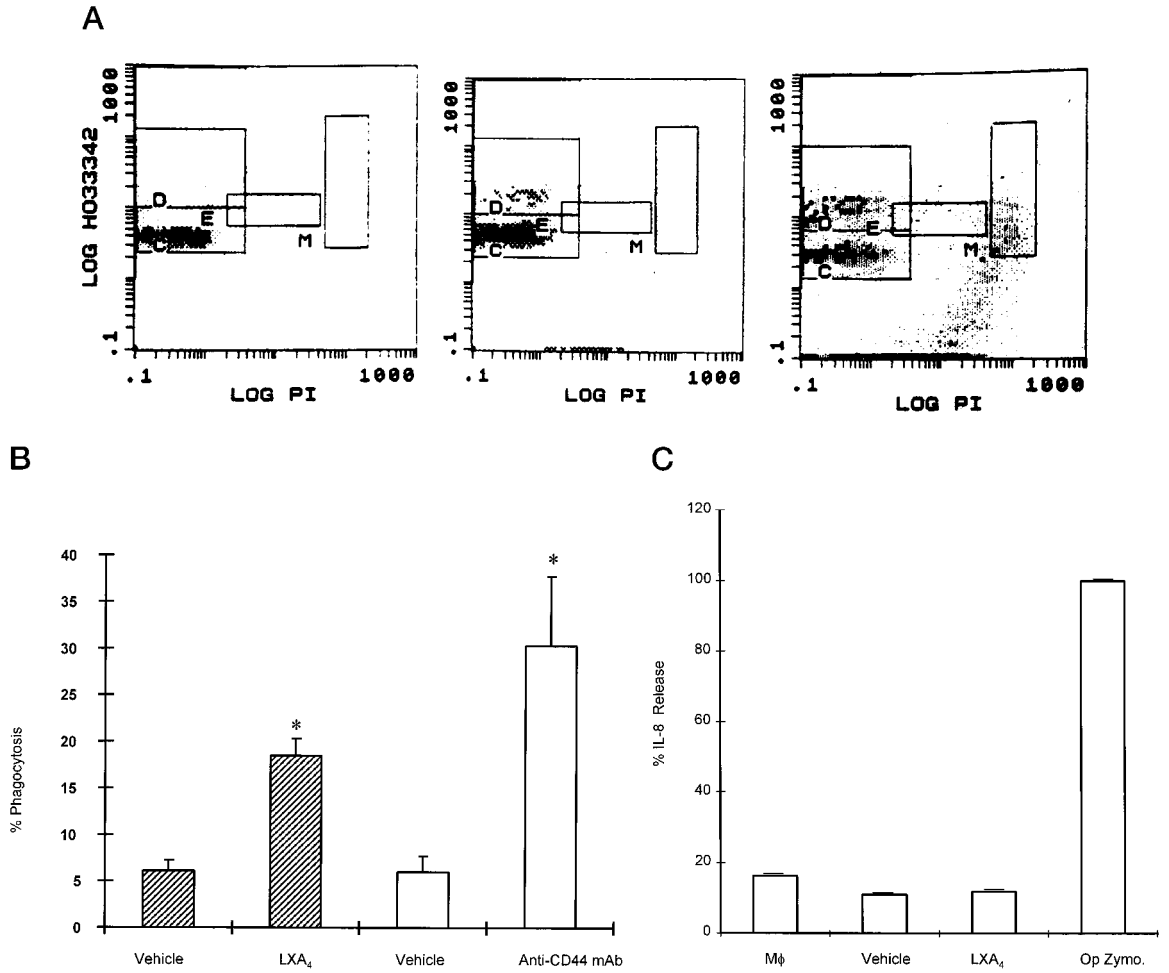

FIGURE 1. LXA_4 rapidly stimulates nonphlogistic phagocytosis of apoptotic PMN by Mϕ. *A*, Dual laser flow cytometry demonstrating progression of PMN from normal (gate C) through early apoptotic (gate D) to late apoptotic/secondary necrotic (gate M) phases during aging for 4 h (*left*), 24 h (*middle*), and 48 h (*right*) (14). For Mϕ phagocytosis assays, PMN were aged for 24 h. *B*, Exposure of Mϕ to LXA_4 (10^{-9} M, 15 min) enhances their phagocytosis of apoptotic PMN during a 30-min coincubation at 37°C. As a positive control in parallel experiments, Mϕ were pretreated with anti-CD44 mAb (J-173, 80 μg/ml, 20 min, 22°C). Data are means ± SEM and are expressed as percent Mϕ staining positively for MPO ($n = 10$, $p < 0.01$). *C*, IL-8 release from Mϕ-PMN coincubations. IL-8 was determined by ELISA (R&D Systems) of supernatants harvested after 30 min from Mϕ cultures, cocultures of aged PMN with Mϕ pretreated with LXA_4 (1 nM) or vehicle (15 min), or Mϕ exposed to opsonized zymosan (10 mg/ml). Data are means ± SEM ($n = 5$) and are expressed as percent zymosan-stimulated IL-8 release.

of this classic nonsteroidal anti-inflammatory agent (6, 12–15). Together these observations raise the possibility that the LX play dynamic roles in the resolution phase of PMN-mediated inflammation.

In this study, we further expand on the anti-inflammatory actions of LX by determining their effects on Mϕ phagocytosis of apoptotic PMN. We demonstrate that exposure of Mϕ to LX causes rapid enhancement of phagocytosis of apoptotic PMN; this response is concentration dependent, involves multiple adhesion molecules, can be mimicked by stable synthetic LX analogues, and is associated with modulation of protein kinase A (PKA) activity.

Materials and Methods

Leukocyte isolation and culture

Human monocytes and PMN were isolated from peripheral venous blood drawn from healthy volunteers following informed written consent. PMN were isolated by density gradient centrifugation and dextran sedimentation (16). Mϕ were prepared from monocytes collected over Ficoll-Paque as reported previously (17). Adherent monocytes were cultured for 5–7 days in RPMI 1640 supplemented with 10% autologous serum and 1% penicillin-streptomycin.

Induction and monitoring of PMN apoptosis

Spontaneous apoptosis of PMN was achieved by culturing 0.5×10^6–1.5×10^6 PMN/ml for 4–48 h (16). Apoptosis was monitored by a combination of light microscopy and dual laser flow cytometry (Epics Elite flow cytometer, Coulter, Hialeah, FL) using Hoechst 33342 and propidium iodide (16).

Mϕ phagocytosis of apoptotic PMN

Mϕ were exposed to experimental stimuli, washed with RPMI 1640, and coincubated with aged PMN in 24-well tissue culture plates (4×10^6 PMN/ml RPMI 1640/well) at 37°C for 30 min. After coincubation, the cells were washed with PBS, fixed with 2.5% glutaraldehyde, and stained for myeloperoxidase (MPO) activity with dimethoxybenzidine in the presence of hydrogen peroxide. Mϕ were routinely negative for peroxidase staining. For each experiment, the number of Mϕ containing one or more PMN was counted by two independent observers in at least five fields (minimum of 400 cells) and expressed as a percentage of the total number of Mϕ in duplicate wells. In initial experiments, phagocytosis of apoptotic PMN was confirmed by electron microscopy.

Determination of IL-8 and monocyte chemoattractant protein-1 (MCP-1) release

IL-8 and MCP-1 were assayed in supernatants of PMN-Mϕ cocultures by ELISA according to the manufacturer's instruction (R&D Systems, Minneapolis, MN).

Determination of PKA activity

PKA activity was determined by a Non-Radioactive PepTag assay (Promega, Madison, WI) using a fluorescent peptide substrate specific for

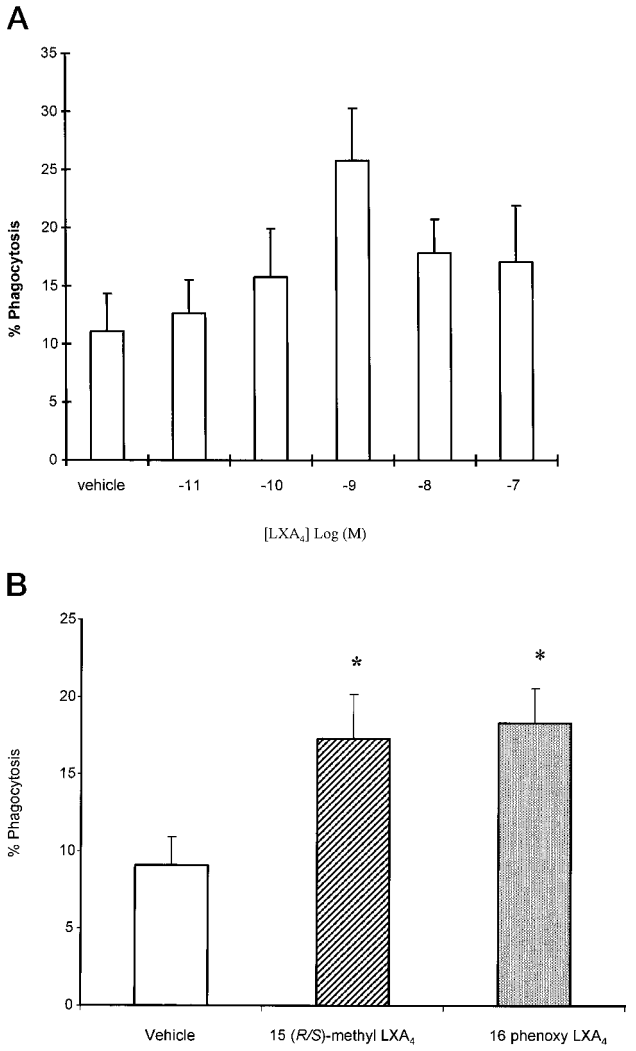

FIGURE 2. LXA_4-triggered phagocytosis of apoptotic PMN by Mϕ is concentration dependent and mimicked by stable synthetic LX analogues. *A*, Mϕ were pretreated with the indicated concentrations of LXA_4 for 15 min and phagocytosis was determined as described for Fig. 1. Data are means ± SEM ($n = 5$) and are expressed as percent Mϕ staining positively for MPO. *B*, Mϕ were pretreated with the stable LX analogues 15-(*R,S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 (10^{-11} M), and phagocytosis was assayed as described above. Data are means ± SEM ($n = 5$, $*$, $p < 0.05$).

PKA-dependent phosphorylation. Mϕ were pretreated with isobutylmethylxanthine (250 μM in RPMI 1640, 15 min), washed once with RPMI 1640, treated with either LXA_4 (10^{-9} M in RPMI 1640 containing 250 μM isobutylmethylxanthine, 15 min) or vehicle, and then stimulated with either PGE_2 (10^{-5} M, 15 min), forskolin (10^{-5} M, 15 min), or diluent at 37°C. Lysates were harvested and PKA activity was assayed. Phosphorylated and unphosphorylated substrates were resolved by agarose gel electrophoresis.

Statistics

Results are expressed as means ± SEM. Statistical significance was determined by Student's *t* test.

Materials

Anti-CD18 mouse mAb (MHM 23) was purchased from Dako (Cambridge, U.K.), anti-$\alpha_v\beta_3$ mouse mAb (23C6) from Serotec (Oxford, U.K.), and anti-CD44 mouse mAb (J-173), FITC-conjugated anti-CD36 (FA6-152) and anti-$\alpha_v\beta_3$ (anti-CD51/61)(AMF-7) mAbs from Beckman Coulter (Luton, U.K.). LXA_4 was obtained from Cascade Biologicals (Berkshire, U.K.). The stable LXA_4 analogues 15-(*R,S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 were prepared by total organic synthesis (18).

Table I. *Influence of arachidonic acid and lipoxygenase-derived eicosanoids on Mϕ phagocytosis of apoptotic PMN*[a]

Experimental Conditions	% Phagocytosis
Vehicle	10.4 ± 1.9
LXA_4	22.3 ± 1.3*
Arachidonic acid	11.9 ± 2.3
15-(*S*)-HETE	13.3 ± 2.4
Leukotriene B_4	12.9 ± 0.8

[a] Mϕ were treated with arachidonic acid, LXA_4, 15-(*S*)-hydroxyeicosatetraenoic acid (15-(*S*)-HETE), or leukotriene-B_4, each at 1 nM for 15 min at 37°C, prior to coincubation with aged PMN for 30 min. Data are means ± SEM ($n = 3$).
$*$, $p < 0.01$ vs vehicle.

Results and Discussion

An important determinant of the resolution of inflammation is the nonphlogistic clearance of apoptotic leukocytes by phagocytosis. Prolonged exposure of cultured human monocyte-derived Mϕ to several cytokines, namely, GM-CSF, TNF-α, IFN-γ, IL-1, and IL-10, enhances their capacity to phagocytose apoptotic PMN in vitro, suggesting that this process is dynamically regulated within inflamed tissue (4). Recent work has shown that exposure of macrophages to corticosteroids enhances their phagocytic capacity by a cycloheximide-sensitive process, raising the intriguing possibility that these agents may suppress inflammation, at least in part, by promoting clearance of PMN (5). Rapidly acting endogenous modulators of phagocytosis in this context remain relatively enigmatic. In the present study, we have investigated whether LX, endogenously produced eicosanoids with anti-inflammatory activities, could influence this process.

LXA_4 stimulates nonphlogistic phagocytosis of apoptotic PMN

PMN undergo spontaneous apoptosis during aging in vitro. This process is characterized morphologically by progression through an initial apoptotic phase typified by chromatin condensation and coalescence of nuclear lobes to a later apoptotic phase characterized by nuclear degradation, evanescence, and secondary necrosis (16). For phagocytosis assays, PMN were studied after 24 h in

Table II. *Mϕ phagocytosis of apoptotic PMN: adhesion requirements*[a]

Experimental Conditions	% Phagocytosis
Vehicle	13.5 ± 4.9
Vehicle + anti-CD36 mAb	7.4 ± 1.7
LXA_4	23.5 ± 6.1
LXA_4 + anti-CD36 mAb	8.4 ± 2.6*
Vehicle	12.8 ± 3.2
Vehicle + anti-CD51/61 mAb	12.2 ± 3.3
LXA_4	24.8 ± 3.0
LXA_4 + anti-CD51/61 mAb	15.9 ± 5.4*
Vehicle	13.2 ± 5.7
Vehicle + anti-CD18 mAb	9.7 ± 2.1
LXA_4	24.3 ± 4.8
LXA_4 + anti-CD18 mAb	8.9 ± 2.5*
Vehicle	3.6 ± 0.5
Vehicle + anti-CD36, CD51/61, and CD18 mAb	2.9 ± 0.1
LXA_4	12.7 ± 1.1
LXA_4 + anti-CD36, CD51/61, and CD18 mAb	4.3 ± 0.4

[a] Mϕ were incubated with mAb for 20 min on ice prior to exposure to LXA_4 (10 nM, 37°C, 15 min) or diluent, followed by coincubation with aged PMN (30 min, 37°C). Anti-CD51/61 (23C6) and anti-CD18 mAb (MHM 23) were purified immunoglobulin from tissue culture supernatants and used at a final concentration of 20 μg/ml and 10 μg/ml, respectively. Anti-CD36 (SMϕ) was ascitic fluid used at a dilution of 125 μl/ml. Data are means ± SEM ($n = 4$).
$*$, $p < 0.05$, by comparison with LXA_4 alone.

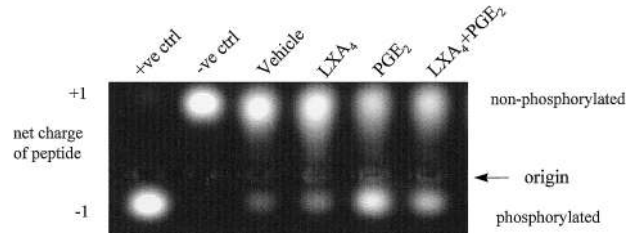

FIGURE 3. LXA_4 inhibits PGE_2-stimulated PKA activation in human monocyte-derived Mϕ. Mϕ were pretreated with LXA_4 (1 nM, 15 min.) or vehicle followed by exposure to either PGE_2 (10 μM) or vehicle (15 min). PKA activity was assayed using the Non-Radioactive PepTag kit (Promega). These results are representative of five experiments, each conducted in duplicate.

culture, a time point at which 25% were in the initial phase of apoptosis and <3% had undergone secondary necrosis as monitored by dual laser flow cytometry (Fig. 1*A*). Pretreatment of Mϕ with LXA_4 (1 nM, 15 min, 37°C) resulted in a 3-fold increase in MPO-positive Mϕ (Fig. 1*B*). In parallel experiments, we included Mϕ pretreated with anti-CD44 mAb (J-173, 80 μg/ml, 20 min, 22°C) before the addition of aged PMN as a positive control (19). Consistent with published data CD44 receptor cross-linking augmented phagocytosis of apoptotic cells (Fig. 1*B*) (19). PMN uptake was not observed with freshly isolated PMN (data not shown).

To facilitate the resolution of inflammation, it is desirable that clearance of apoptotic cells does not provoke the release of proinflammatory mediators from phagocytes (1–3, 20). Indeed, recent work has shown active suppression of proinflammatory cytokine production during phagocytosis of apoptotic cells (21). To investigate whether LX-mediated phagocytosis of apoptotic PMN is nonphlogistic, we assayed release of the prototypic proinflammatory cytokines IL-8 and MCP-1 in supernatants of coincubations LXA_4-treated Mϕ and aged PMN. LXA_4-stimulated phagocytosis was not associated with increased IL-8 release by comparison with Mϕ phagocytosis of opsonized zymosan (Fig. 1*C*). Furthermore, LX-stimulated phagocytosis of PMN did not provoke MCP-1 release (data not shown). Interestingly, LXA_4 and its analogues have previously been shown to inhibit release of cytokines and chemokines including IL-8 in other cell types (9).

LXA_4-mediated phagocytosis of apoptotic PMN is concentration dependent, specific, and mimicked by stable LXA_4 analogues

LXA_4-triggered phagocytosis was concentration dependent (EC_{50} $\sim 0.5 \times 10^{-9}$ M; Fig. 2*A*); this value is consistent with the reported K_d of the cloned LXA_4 receptor which is expressed by Mϕ (22). The specificity of the effect of LXA_4 relative to other eicosanoids was investigated. LX-augmented phagocytosis was not mimicked by exposure of Mϕ to either the LX precursors arachidonic acid (10^{-9} M) or 15(*S*)-hydroxyeicosatetraenoic acid (10^{-9} M), or by exposure to the proinflammatory product of the 5-lipoxygenase pathway, leukotriene B_4 (1 nM; Table I), or to PGE_2 (1 nM; data not shown). LXA_4 is metabolized rapidly via pathways initially involving dehydrogenation at carbon-15. To circumvent such degradation, a panel of synthetic, stable LXA_4 analogues have been designed (18). These analogues act as ligands for the human myeloid LXA_4 receptor and retain the ability of the native compound to inhibit PMN-endothelial cell adhesion and PMN recruitment in vitro and in vivo (12, 13, 18, 22). We investigated whether the stable synthetic LXA_4 analogues 15-(*R,S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 could mimic the effects of the native compound on Mϕ phagocytosis. Both analogues stimulated Mϕ phagocytosis of apoptotic PMN at picomolar concentrations (Fig. 2*B*). The potency of the analogues relative to the native compound are remarkable given the previously described rapid inactivation of LXA_4 by monocytes (22). The data with 15-(*R,S*)-methyl-LXA_4 are particularly interesting as this is a racemate of both native LXA_4 and aspirin-triggered 15-epi-LXA_4 (12, 13). Thus, acceleration of PMN clearance is a potential component of aspirin-related bioactivities within a local inflammatory milieu.

Table III. *Mϕ phagocytosis of apoptotic PMN: role of cAMP and PKA*[a]

Experimental Conditions	% Phagocytosis
Vehicle	9.8 ± 1.2
LXA_4	15.4 ± 1.6*
8-bromo-cAMP + vehicle	5.1 ± 2.6
8-bromo-cAMP + LXA_4	5.5 ± 1.7
Vehicle	10.4 ± 1.9
LXA_4	22.3 ± 1.3*
Rp-cAMP + vehicle	19.1 ± 2.3*
Rp-cAMP + LXA_4	22.1 ± 1.6*

[a] Mϕ were treated with either LXA_4 (1 nM), 8-bromo-cAMP (2 mM) or Rp-cAMP (100 μM) alone or in combination for 15 min at 37°C prior to coincubation with aged PMN for 30 min. Data are means ± SEM (n = 3).

*, $p < 0.05$ vs vehicle.

LXA_4-stimulated phagocytosis of apoptotic PMN: adhesion requirements

Mϕ recognize apoptotic cells via several mechanisms, including integrins, phosphatidylserine recognition systems, lectins, and scavenger receptors, frequently acting in concert (23–27). In the present study, treatment of Mϕ with mAbs against either CD36 or $\alpha_v\beta_3$ blocked phagocytosis of apoptotic PMN induced by LXA_4 (Table II), indicating a role for the $\alpha_v\beta_3$-CD36 complex in LXA_4-stimulated phagocytosis. In parallel experiments, treatment of Mϕ with LXA_4 (10^{-9} M, 15 min) did not alter cell surface expression of either $\alpha_v\beta_3$ or CD36, as determined by flow cytometry (n = 3; data not shown). These results suggest that LXA_4 promotes Mϕ phagocytosis of apoptotic PMN either by increasing the avidity of the $\alpha_v\beta_3$-CD36 complex for PMN ligands or by influencing subsequent cytoskeletal events that are dependent on initial macrophage-PMN adhesion. There is compelling evidence that a Mϕ adhesion complex involving the CD36 scavenger receptor and $\alpha_v\beta_3$ integrin (CD51/61, vitronectin receptor) plays a central role in the recognition of apoptotic PMN (23–28).

LXA_4-triggered phagocytosis was also inhibited by anti-CD18 mAb (Table II), indicating the involvement of other adhesion ligands acting either in parallel or in sequence. The finding that anti-CD18 mAb attenuates LXA_4-triggered phagocytosis is noteworthy for several additional reasons. This response distinguishes LXA_4-triggered phagocytosis of apoptotic PMN from rapid PMN uptake stimulated by ligation of Mϕ CD44 which is not CD18 dependent (19). In addition, this result highlights the different effects of LXA_4 on the adhesive functions of macrophages and PMN, LXA_4 being a robust stimulus for CD11/CD18-dependent macrophage phagocytosis of PMN in the present study and a potent inhibitor of CD11/CD18-dependent PMN-endothelial cell adhesion and transmigration in our previous studies (7).

LXA_4-stimulated phagocytosis and PKA activity

Mϕ high-affinity LXA_4 receptors have previously been shown to be coupled through pertussis toxin (PTX)-sensitive G proteins (22). In the present study, prior exposure of Mϕ to pertussis toxin (200 ng/ml, 18 h) inhibited phagocytosis (percent phagocytosis: vehicle, 11.0 ± 1.3; LXA_4, 20.8 ± 5.4; PTX alone, 5.1 ± 0.8; and LXA_4 plus PTX, 4.5 ± 0.8, n = 3), consistent with a receptor-

mediated response involving G_i proteins. Elevation of intracellular cAMP by prior exposure of Mϕ with the cell permeant analogue 8-bromo-cAMP inhibited LX-stimulated phagocytosis and, conversely, the PKA inhibitor Rp-cAMP mimicked the effects of LXA_4 (Table III). Interestingly, the effects of Rp-cAMP and LXA_4 on promoting phagocytosis were not additive (Table III), suggesting that they may act at a common target. This observation was further characterized by direct assay of PKA activity. Exposure of macrophages to LXA_4 (10^{-9} M) consistently blunted PKA activation induced by addition of exogenous PGE_2 (10^{-5} M, 15 min, $n = 5$; Fig. 3) and by forskolin (10^{-5} M, 15 min, $n = 5$; data not shown), known activators of Mϕ adenylyl cyclase. These data are of interest in the context of cAMP-dependent regulation of cytoskeletal functions such as F-actin assembly, cell adhesion, and cell spreading. Recent data from others have shown that increased intracellular cAMP is associated with decreased Mϕ phagocytosis of apoptotic cells, reduced Mϕ adhesiveness, and a perturbation in actin and talin colocalization at contact points (29). Our data showing blockade of Mϕ phagocytosis of apoptotic PMN with anti-CD36 mAb is particularly interesting given that CD36 is a PKA substrate (30). Platelet CD36 is constitutively phosphorylated and its dephosphorylation is associated with increased cytoadhesion (31). Consistent with the hypothesis that LX-mediated protein dephosphorylation is an important determinant of phagocyte-apoptotic cell recognition are our preliminary observations that the phosphatase inhibitor okadaic acid blocks LXA_4-stimulated phagocytosis (data not shown).

In conclusion, our results demonstrate that Mϕ phagocytosis of apoptotic PMN is accelerated by the endogenous lipoxygenase-derived lipid mediator LXA_4. This bioactivity was observed at nanomolar concentrations, and is thus likely to be biologically relevant in vivo, and was also evoked by stable LXA_4 analogues at picomolar concentrations. When viewed in the context of the ability of LXA_4 and its analogues to reduce the intensity of inflammatory infiltrates and tissue injury in experimental models of inflammation, these observations highlight the attractiveness of the LX network as an endogenous anti-inflammatory system that could be harnessed pharmacologically for therapeutic gain.

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