Lipoxins, Aspirin-Triggered Epi-Lipoxins, Lipoxin Stable Analogues, and the Resolution of Inflammation: Stimulation of Macrophage Phagocytosis of Apoptotic Neutrophils In Vivo

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Abstract. Lipoxins (LX) are eicosanoids with antiinflammatory activity in glomerulonephritis (GN) and inflammatory diseases, hypersensitivity, and ischemia reperfusion injury. It has been demonstrated that LXA₄ stimulates non-phlogistic phagocytosis of apoptotic polymorphonuclear neutrophils (PMN) by monocyte-derived macrophages (MΦ) in vitro, suggesting a role for LX as endogenous pro-resolution lipid mediators. It is here reported that LXA₄, LXB₄, the aspirin-triggered LX (ATL) epimer, 15-epi-LXB₄, and a stable synthetic analogue 15(R/S)-methyl-LXA₄ stimulate phagocytosis of exogenously administered excess apoptotic PMN by macrophages (MΦ) in vivo in a classic model of acute inflammation, namely thioglycollate-induced peritonitis. Significant enhancement of phagocytosis in vivo was observed with 15-min exposure to LX and with intraperitoneal doses of LXA₄, LXB₄, 15(R/S)-methyl-LXA₄, and 15-epi-LXB₄ of 2.5 to 10 μg/kg. Non-phlogistic LX-stimulated phagocytosis by MΦ was sensitive to inhibition of PKC and PI 3-kinase and associated with increased production of transforming growth factor–β₁ (TGF-β₁). LX-stimulated phagocytosis was not inhibited by phosphatidylinerse receptor (PSR) antisera and was abolished by prior exposure of MΦ to β1,3-glucan, suggesting an endogenous MΦ-PMN recognition mechanism. Interestingly, the recently described peptide agonists of the LXA₄ receptor (MYFINITL and LESIFRSLFRVM) stimulated phagocytosis through a process associated with increased TGF-β₁ release. These data provide the first demonstration that LXA₄, LXB₄, ATL, and LX stable analogues rapidly promote MΦ phagocytosis of PMN in vivo and support a role for LX as rapidly acting, pro-resolution signals in inflammation. Engagement of the LXR by LX generated during cell-cell interactions in inflammation and by endogenous LXR peptide agonists released from distressed cells may be an important stimulus for clearance of apoptotic cells and may be amenable to pharmacologic mimicry for therapeutic gain.

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Rapid, efficient and tightly regulated recruitment and clearance of polymorphonuclear neutrophil (PMN) at sites of inflammation are essential components of effective host defense. Evidence from in vitro models and from histopathology suggests that tissue damage mediated by PMN is limited by apoptosis and subsequent phagocytosis of the apoptotic PMN by macrophages (MΦ) and “nonprofessional” phagocytes (1). A direct role for PMN in tissue injury in inflammation and ischemia reperfusion injury of the kidney and other organs is well established (2). Impaired clearance of apoptotic cells by MΦ has been implicated in the pathogenesis of chronic inflammatory conditions, including glomerulonephritis (GN) and systemic lupus erythematosus (SLE) (3). The endogenous signals that promote clearance of apoptotic PMN from an inflammatory focus are still being defined. By dissecting out the mediator systems that regulate this process, it may be possible to design new pro-resolution strategies for inflammatory diseases.

Lipoxins (LX), an acronym for lipoxygenase interaction products, are endogenously produced eicosanoids with anti-inflammatory actions in GN and other diseases (4). LX are formed principally by transcellular metabolism initiated by sequential oxygenation of arachidonic acid by either 15- and 5-lipoxygenases or 5- and 12-lipoxygenases (5). Lipoxin bio-
synthesis has been demonstrated in a range of inflammatory diseases, including experimental immune complex glomerulonephritis in rats and mice (6,8,9). Platelet-PMN biosynthetic interactions provide the dominant pathway for LX formation in the initial stages of immune complex–mediated glomerulonephritis and are likely superseded by leukocyte interactions with cytokine-activated resident tissue cells as the inflammatory process evolves (9,10). LX were originally proposed to act as “braking signals” in inflammation on the basis of their ability to inhibit neutrophil chemotaxis, adhesion, and transmigration across endothelia and epithelia (reviewed in reference 4). LXA₄ and LXB₄ inhibit LTB₄-induced PMN chemotaxis and PMN-endothelial cell adhesion supported by CD11/CD18 integrins (7). LXA₄ and LXB₄ also attenuate LTC₄- and LTD₄-triggered endothelial hyperadhesiveness for PMN, in part by inhibiting mobilization of P-selectin from Weibel Palade bodies to the endothelial cell surface (6). More recently, the repertoire of antiinflammatory actions of LX has been expanded to include attenuation of cytokine and chemokine release (11,12), synoviocyte metalloproteinases (13), eosinophil trafficking (14), inhibition of mesangial cell proliferation (15), and stimulation of phagocytosis of apoptotic PMN in vitro (16).

Lipoxin A₄ [(5S,6R,15S)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid] and its positional isomer lipoxin B₄ [(5S,14R,15S)-trihydroxy-6,8,12-trans-10-cis-eicosatetraenoic acid] are the two principal species formed in mammalian cells (reviewed in reference 4). The rapid metabolic degradation of native LX by dehydrogenation at C15 and possibly ω-oxidation at C20 has prompted the design and synthesis of stable analogues, which retain many of the properties of the native LX, including inhibition of PMN adhesion and transmigration (17,4). In a cytokine primed milieu, the catalytic activity of aspirin-acetylated cyclooxygenase type 2 (COX-2) is shifted from a cyclooxygenase to 15R lipoxygenase. In this context, 15(R)-HETE is produced and can then be metabolized to 15 epimers of LXA₄ and LXB₄ during cell-cell interactions (18). These aspirin-triggered lipoxins (ATL) also possess antiinflammatory activity (19,20).

Here we demonstrate that LX, ATL, 15-epi-LXB₄, and the stable synthetic LX analogue 15(R/S)-methyl-LXA₄ promote Mϕ phagocytosis of apoptotic PMN in vivo. We expand on the signal transduction pathways mediating LX-stimulated phagocytosis and further define the adhesive requirements necessary for efficient phagocytosis. We demonstrate that recently described peptide agonists of the lipoxin receptor (ALXR) can also promote non-phlogistic phagocytosis of apoptotic PMN associated with transforming growth factor–β₁ (TGF-β₁) release. These data expand on the antiinflammatory repertoire of LX and provide the first evidence of rapid regulation of PMN clearance by endogenous lipid mediators in vivo. This raises the possibility that such a role may be played not only by lipoxigenase-derived lipid mediators but also by necrotactic peptides released by dying cells. The data suggest that it may be possible to actively promote the resolution of inflammatory disease by harnessing the system pharmacologically.

**Materials and Methods**

**Materials**

RPMI 1640 and phosphate-buffered saline (PBS) were obtained from Life Technologies (Grand Island, NY). LXA₄ and LXB₄ were obtained from Cascade Biologicals (Berkshire, UK). 15(R/S)-methyl-LXA₄, 16-phenoxy-LXA₄ and the aspirin-triggered 15-epi-LXB₄ were prepared by total organic synthesis (21). CM-orange (CMO, cell tracker orange) was from Molecular Probes (Oregon, US). Rat cyto
tokines tumor necrosis factor–α (TNF-α), TGF-β, interferon-γ (IFN-γ), and TGF-β₁ and MCP-1 human enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (Oxon, UK). The anti-CD36 monoclonal antibody SMφ (IgM) was provided by Dr. Nancy Hogg (ICRF, London) (22). Anti-α₁β₁ mouse monoclonal antibody (23C6) was purchased from Serotec (Oxford, UK). FITC-conjugated anti-CD36 (FA6–152) and anti-α₁β₁ (anti-CD51/61) (AMF-7) monoclonal antibodies were from Beckman Coulter (Luton, UK). Phosphatidylserine receptor (PSR) antisera were a gift from Dr. Valerie Fadok (National Jewish Medical and Research Center, Denver, CO) (23); isotype-matched control IgM was obtained from Cal
biochem (Nottingham, UK). β1,3-glucan was purchased from Accurate Chemicals (Westbury, NY). The MHC-binding peptide (MYFINILTL) (MHC bp) and MMK-1 peptide (LESIFRSLLFRVM) were gifts from Prof. Charles N. Serhan (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA.) (19). All other chemicals were obtained from Sigma (Poole, UK).

**Human Leukocyte Preparation and Culture**

Human monocytes and PMN were isolated from peripheral venous blood drawn from healthy volunteers who had provided informed written consent subsequent to approval from institutional ethics committees. PMN were isolated by density gradient centrifugation and dextran sedimentation and aged for 24 h; apoptosis was verified by flow cytometry as previously documented (16). Mϕ were prepared from monocytes collected over Ficoll-Paque as previously reported (16). Adherent monocytes were cultured for 5 to 7 d in RPMI supplemented with 10% autologous serum and 1% penicillin-streptomycin.

**Mϕ Phagocytosis of Apoptotic PMN In Vitro**

Mϕ were treated with reagents as indicated and washed with RPMI 1640 before coincubation with aged PMN in 24-well tissue culture plates (4 × 10⁶ PMN/ml of RPMI per well) at 37°C for 30 min. Phagocytosis was assayed by myeloperoxidase (MPO) staining of co-cultures fixed with 2.5% glutaraldehyde as previously reported (16). Mϕ were routinely negative for peroxidase staining. 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 in duplicate wells. Phagocytosis of apoptotic PMN was confirmed by electron microscopy.

**Electron Microscopy**

The coincubation cultures was washed with PBS, detached, and centrifuged at 500 × g for 15 min. The cell pellet was fixed in 2.5% glutaraldehyde in PBS for 1 h at room temperature. Glutaraldehyde was removed, and the pellet was washed three times in 6.8% sucrose/PBS. One percent osmium tetroxide was then added for 1 h at room temperature before a washing step with 6.8% sucrose/PBS. The pellet was then dehydrated in ascending grades of ethanol and embedded in epoxy resin at 60°C. For light microscopy, 1-µm sections were cut with a glass knife and stained with tolueene blue. Selected areas were trimmed, and ultrathin sections were cut at 50 nm using a diamond
knife; sections were picked up on 100 mesh copper grids and stained with uranyl acetate and lead citrate. These were examined and photographed in a JOEL 2000 EX Temscan at 80KV and using an objective aperture of 100 µm.

**Inhibitor and Antibody Studies**

Mφ were washed with RPMI 1640 before treatments with the stable cell-permeable cAMP analogue 8-Br-cAMP (2 mM) (24), the protein kinase A inhibitor Rp-cAMP (100 µM) (16), the PI 3-kinase inhibitor LY 294002 (10 µM) (15), the PKC inhibitor GF 10230X (10 µM) (15), the MAP kinase inhibitor PD 98059 (20 µM) (15), or appropriate vehicles for 15 min at 37°C before stimulation with LXA₄ or LXB₄ (1 nM; 15 min; 37°C). The treated cells were washed with RPMI 1640 before coincubation with aged PMN and phagocytosis assayed as described above.

Antibody blockade of cell surface adhesion molecules was affected by exposure of Mφ to anti-CD5/61 mAb (20 µg/ml) and anti-CD36 mAb (SMα) ascitic fluid (22) (125 µl/ml) on ice for 20 min. Mφ were then treated with LX (1 nM; 15 min; 37°C) or control before coincubation with apoptotic PMN for 30 min, and phagocytosis was assayed as above.

In experiments investigating the role of the phosphatidylserine receptor (PSR), Mφ were treated with PSR antisera (200 µg/ml) and isotype control human IgM on ice for 30 min before treatment with LX and subsequent coincubation with PMN (23). Phagocytosis was assessed by staining the co-cultures for MPO activity as above.

**Induction of the PS Recognition System in Mφ**

Treatment of Mφ with β1,3-glucan confers PSR dependence on Mφ phagocytosis of apoptotic PMN (25). On day 5, human Mφ were treated with β1,3-glucan (25 µg/ml) for 48 h before washing twice with RPMI before treatments with LX assay of phagocytosis.

**Rat Bone Marrow–Derived Macrophages: Isolation and Cell Culture**

Rat bone marrow–derived Mφ (BMDM) were isolated by standard procedures (26). Briefly, bone marrow cells from the dissected femurs of male Sprague-Dawley rats were flamed with complete medium through a 25-gauge needle to form a single-cell suspension. Cells were cultured in 75-mm tissue culture flasks in Dulbecco’s modified Eagle medium (DMEM) containing 2 mM glutamine, 10% fetal calf serum (FCS), 1% penicillin-streptomycin, and 10% conditioned medium from L929 cells as a source of macrophage-colony stimulating factor (M-CSF). After 7 days, cells were plated into 24-well plates at a concentration of 5 × 10⁵ cells/well and rested for 24 h in M-CSF–free medium. Cells were stimulated with murine TNF-α (10 ng/ml for 24 h), IFN-γ/TNF-α (10 ng/ml for 4 h and 10 ng/ml for 20 h), or TGF-β (5 ng/ml for 24 h) before LXA₄ treatment (1 nM for 15 min). Phagocytosis of aged human PMN was assayed as above.

**Assay of TGF-β₁ and MCP-1 Release from Mφ**

TGF-β₁ and MCP-1 were assayed in supernatants from co-cultures of PMN-Mφ by ELISA according to the manufacturer’s instruction (R&D systems).

**Assessment of LX-Stimulated Mφ Phagocytosis of Apoptotic PMN In Vivo**

Ten-week-old BALB/C mice were housed and treated under UK Home Office-approved conditions in University of Edinburgh, Faculty of Medicine animal facility. An enriched population of inflammatory Mφ was induced in the peritoneal cavity of groups of male BALB/C adult mice by a single intraperitoneal injection of 1 ml of 3% (wt/vol) thioglycollate 5 d before assay (3). On day 4, human PMN were isolated from peripheral blood of healthy volunteers. PMN were labeled with CM-orange (CMO, cell tracker orange) and aged in culture for 24 h to render a portion apoptotic. LXA₄, LXB₄, 15(R/S)-methyl-LXA₄, 16-phenoxy-LXA₄, and 15-epi-LXB₄ were administered intraperitoneally 15 min before instillation of a bolus of labeled, apoptotic PMN (approximate 3:1 ratio of PMN to Mφ) (3). After 30 min, mice were sacrificed and peritoneal cells were harvested by lavage. Cytospin preparations of the lavages were fixed, and the number of Mφ containing ingested CMO-positive, apoptotic PMN was determined by fluorescence microscopy and expressed relative to total number of Mφ recovered.

**Statistics**

Statistical significance was determined by t tests or one-way ANOVA where appropriate.

**Results**

**Both Lipid and Peptide Agonists for LX Receptors Stimulate Non-Phlogistic Phagocytosis of Apoptotic PMN**

Figure 1A shows LX-stimulated Mφ phagocytosis of apoptotic PMN as demonstrated by electron microscopy. As previously reported for LXA₄ in vitro (16), LXB₄ stimulated phagocytosis of apoptotic PMN in this model system, indicating that this pro-resolution effect is shared by the two major LX generated endogenously in mammalian systems. LXA₄ and LXB₄ demonstrated similar dose-response relationships with an EC₅₀ of 0.5 × 10⁻⁹ M and a maximum stimulation observed at 1 nM (Figure 1B). Interestingly, additivity was not observed with simultaneous exposure of Mφ to LXA₄ and LXB₄ (data not shown), indicating the possibility of a common signaling pathway resulting in enhanced phagocytosis. Aspirin acetylation of cyclooxygenase-2 (COX-2) promotes the formation of 15 epoxy-LX in the context of neutrophil-endothelial cell interactions. The aspirin-triggered epi lipoxins (ATL) are implicated in the antiinflammatory actions of aspirin in vivo. In this study, the ATL 15 epi-LXA₄ also stimulated macrophage phagocytosis (Figure 1B). Recent data indicate that, in addition to LXA₄ and the stable synthetic analogues binding to the ALXR, specific peptides can also bind and elicit responses (19,27). We assessed the effects of such agents on stimulation of Mφ phagocytosis of apoptotic PMN. Treatment of Mφ for 15 min with either MHC bp or MMK-1 at 1 nM significantly enhanced phagocytosis (2-fold and 1.7-fold, respectively) in vitro, and these actions were not additive with LXA₄ (Figure 1C) when used at a suboptimal concentration (0.5 nM).

In contrast to phagocytosis of opsonized particles, non- phlogistic Mφ phagocytosis of apoptotic PMN may contribute to the resolution of inflammation, being characteristically coupled with TGF-β₁ release and not associated with release of proinflammatory cytokines such as IL-8 and MCP-1 (28). To investigate whether peptide and LX-mediated phagocytosis might promote the resolution of inflammation, Mφ were treated with LXA₄, MHC bp, and MMK-1 (1 nM for 15 min) before coincubation with apoptotic PMN, and TGF-β₁ and...
Figure 1. Lipoxins (LX), aspirin-triggered LX (ATL), and peptide agonists promote non-phlogistic macrophage (Mφ) phagocytosis of apoptotic polymorphonuclear neutrophils (PMN). (A) Mφ were treated with LXA₄ (1 nM for 15 mins) before coincubation with aged PMN for 30 min. Electron microscopy was performed as described in Materials and Methods. (B) LXB₄ and the aspirin-triggered 15-epi-LXB₄ stimulate Mφ phagocytosis. Mφ were treated with LXB₄ (1 nM) or 15-epi-LXB₄ (1 × 10⁻¹¹ M) for 15 min before coincubation with aged PMN for 30 min. Data are fold induction ± SEM (n = 4); *P < 0.05 versus control. (C) Peptide agonists of LXA₄ receptor (ALXR) stimulate Mφ phagocytosis. Mφ were treated with MHC bp or MMK-1 synthetic peptide (1 nM for 15 min) alone or in combination with LXA₄ (1 nM) for 15 min before coincubation with aged PMN for 30 min. Data are mean ± SEM (n = 4); *P < 0.05 versus control. (D) LXA₄ and peptide agonists stimulate the release of transforming growth factor-β₁ (TGF-β₁). Mφ were treated with LXA₄, MHC bp, and MMK-1 (1 nM) for 15 min at 37°C before coincubation with apoptotic PMN. After 30 min, supernatants were collected and TGF-β₁ production was measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean TGF-β₁ production (pg/ml) ± SD. (E) LXA₄ and peptide agonists do not provoke release of MCP-1. Mφ were treated with LXA₄, MHC bp, and MMK-1 (1 nM) for 15 min at 37°C before coincubation with apoptotic PMN. After 30 min, supernatants were collected and MCP-1 production was measured by ELISA. Data are expressed as mean MCP-1 production (pg/ml) ± SD.
Lipoxins Stimulate Phagocytosis In Vivo

Phagocytosis of Exogenously Administered Apoptotic PMN In Vivo

To determine if LX stimulate Mφ phagocytosis of apoptotic PMN in vivo, we employed the mouse (BALB/C) thioglycol-

Table 1. The effect of PKC, PI 3-kinase, and phosphatase inhibition on LX-stimulated phagocytosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Increase in Phagocytosis ± SEM</th>
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</thead>
<tbody>
<tr>
<td>LXB₄</td>
<td>84.8 ± 2.0</td>
</tr>
<tr>
<td>GF</td>
<td>4.0 ± 0.9b</td>
</tr>
<tr>
<td>GF 109203X + LXB₄</td>
<td>4.0 ± 1.8b</td>
</tr>
<tr>
<td>LXB₄</td>
<td>141.4 ± 7.1</td>
</tr>
<tr>
<td>LY 294002</td>
<td>31.0 ± 3.8b</td>
</tr>
<tr>
<td>LY 294002 + LXB₄</td>
<td>68.9 ± 4.0b</td>
</tr>
<tr>
<td>LXB₄</td>
<td>87.5 ± 11.6</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>16.5 ± 10.4b</td>
</tr>
<tr>
<td>Okadaic acid + LXB₄</td>
<td>5.8 ± 7.2b</td>
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</table>

a Mφ were treated with GF 109203X (20 μM), LY 294002 (10 μM), and Okadaic acid (100 nM) for 15 min at 37°C before stimulation with LXB₄ (1 nM for 15 mins at 37°C). Data are % increase in phagocytosis versus control ± SEM (n = 5).
b P < 0.05 versus control.

In the present study, LXB₄-stimulated phagocytosis, similar to the LXA₄-induced response (16), was inhibited by mAbs against Mφ CD36 and αβ₂ integrins (control, 14.5 ± 3.25; LXB₄, 28.6 ± 6.16;* anti-CD36 mAb, 13.93 ± 3.49; anti-CD36 mAb + LXB₄, 14.60 ± 4.44; anti-CD51/61 mAb, 14.10 ± 1.83; anti-CD51/61 mAb + LXB₄, 12.58 ± 2.36;% phagocytosis ± SEM; n = 3; * P < 0.05 versus control). The Mφ PSR is an important molecule in Mφ recognition of apoptotic cells in many systems (23). Here we evaluated the role of the PSR in LXA₄ and LXB₄-stimulated phagocytosis using PSR antisera. PSR antisera (200 μg/ml) did not inhibit LX-stimulated phagocytosis (Figure 2A). Exposure of Mφ to β1,3-glucan renders Mφ-PMN interaction PSR-dependent (25). Here we report that treatment of Mφ with 25 μg/ml β1,3-glucan (48 h) abolished both LXA₄ and LXB₄-stimulated phagocytosis (Figure 2B). To verify that both the PSR antisera and β1,3-glucan were functional in our system and capable of eliciting previously reported effects (23,25,28,29), we mea-

Enhancement of Mφ Phagocytosis of Apoptotic PMN

In Vitro: A PSR-Independent Mechanism

In the present study, LXB₄-stimulated phagocytosis, similar to the LXA₄-induced response (16), was inhibited by mAbs against Mφ CD36 and αβ₂ integrins (control, 14.5 ± 3.25; LXB₄, 28.6 ± 6.16;* anti-CD36 mAb, 13.93 ± 3.49; anti-CD36 mAb + LXB₄, 14.60 ± 4.44; anti-CD51/61 mAb, 14.10 ± 1.83; anti-CD51/61 mAb + LXB₄, 12.58 ± 2.36;% phagocytosis ± SEM; n = 3; * P < 0.05 versus control). The Mφ PSR is an important molecule in Mφ recognition of apoptotic cells in many systems (23). Here we evaluated the role of the PSR in LXA₄ and LXB₄-stimulated phagocytosis using PSR antisera. PSR antisera (200 μg/ml) did not inhibit LX-stimulated phagocytosis (Figure 2A). Exposure of Mφ to β1,3-glucan renders Mφ-PMN interaction PSR-dependent (25). Here we report that treatment of Mφ with 25 μg/ml β1,3-glucan (48 h) abolished both LXA₄ and LXB₄-stimulated phagocytosis (Figure 2B). To verify that both the PSR antisera and β1,3-glucan were functional in our system and capable of eliciting previously reported effects (23,25,28,29), we mea-

LX Reprograms BMDM Phenotype

BMDM can be programmed to develop distinct phenotypes in response to exposure to specific cytokines, including TGF-β, IFN-γ/TNF-α, and TNF-α (30). IFN-γ/TNF-α stimulate BMDM to adopt a cytotoxic phenotype characterized by sustained NO production and a diminished phagocytic capacity (30). Exposure of rat BMDM to TNF-α stimulates phagocytosis of apoptotic PMN and is not associated with NO release, characteristic of a reparative phenotype. Here we investigated whether LX could stimulate phagocytosis in such cytokine-primed BMDM. LXA₄ and stable LX analogues significantly stimulated naïve rat BMDM to phagocyte apoptotic PMN (Figure 3A).

After programming by cytokine-programmed cohorts (Figure 3, B and C): TNF-α treatment (10 ng/ml for 24 h) stimulated a significant increase in phagocytosis that was further enhanced by stimulation with LXA₄ (1 × 10⁻⁹ M for 15 min), 15(R/S)-methyl-LXA₄ (1 × 10⁻¹¹ M for 15 min), or 16-phenoxy-LXA₄ (1 × 10⁻¹¹ M for 15 min) (Figure 3B). Exposure to IFN-γ/TNF-α (10 ng/ml for 4 h and 10 ng/ml for 24 h) reduced the phagocytic capacity of BMDM by approximately 25%, which is compatible with previous data (30). Intriguingly, subsequent exposure of such treated MDM to LXA₄ (1 × 10⁻⁹ M for 15 min), 15(R/S)-methyl-LXA₄ (1 × 10⁻¹¹ M for 15 min), or 16-phenoxy-LXA₄ (1 × 10⁻¹¹ M for 15 min) rescued this phenotype and a significant increase in phagocytosis was observed comparable to naïve BMDM (Figure 3C).

LX Stimulate Mφ Phagocytosis of Exogenously Administered Apoptotic PMN In Vivo

To determine if LX stimulate Mφ phagocytosis of apoptotic PMN in vivo, we employed the mouse (BALB/C) thioglyc-
Figure 2. LX-stimulated phagocytosis is phosphatidyserine receptor–independent. (A) Phosphatidyserine receptor (PSR) antisera did not block LX-stimulated phagocytosis. Mφ were treated with PSR antisera (200 μg/ml) or isotype control Ab on ice for 30 min before treatment with LXA₄ (1 nM for 15 mins at 37°C) and subsequent coincubation with apoptotic PMN. Data are mean % phagocytosis ± SEM (n = 3); *P < 0.05. (B) β1,3-glucan treatment abolishes sensitivity of Mφ to LX-stimulated phagocytosis. Mφ were treated with 25 μg/ml β1,3-glucan for 48 h before treatment with LXA₄ and LXB₄ (1 nM for 15 mins at 37°C) and coincubation with apoptotic PMN for 30 min. Data are expressed as mean % phagocytosis ± SEM (n = 4); *P < 0.05 versus control. (C) PSR Ab and β1,3-glucan treatment of Mφ elicits TGF-β₁ release. TGF-β₁ release from Mφ treated with control, PSR Ab (200 μg/ml), isotype control Ab, and β1,3-glucan (25 μg/ml) for 18 h at 37°C was determined by ELISA. Data are expressed as mean cytokine production ± SD.

Figure 3. LX stimulate phagocytosis of bone marrow–derived Mφ (BMDM) under basal conditions and after cytokine programming. (A) BMDM were treated with LXA₄ (1 nM for 24 h), 15(R/S)-methyl-LXA₄, and 16-phenoxy-LXA₄ (1 × 10⁻¹¹ M for 24 h). BMDM were treated with (B) tumor necrosis factor–α (TNF-α) (10 ng/ml for 24 h) or (C) interferon-γ (IFN-γ)/TNF-α (10 ng/ml for 4 h and 10 ng/ml for 20 h) before treatment with LXA₄ (1 nM for 15 mins), 15(R/S)-methyl-LXA₄ or 16-phenoxy-LXA₄ (1 × 10⁻¹¹ M for 15 mins). After 30-min coincubations with aged PMN, phagocytosis was assessed by staining the cocultures for MPO activity. A minimum of 400 cells were counted to determine the number of Mφ that had ingested one or more PMN. Results are expressed as fold/basal phagocytosis ± SEM (n = 4); *P < 0.05 versus control.
late-elicited peritonitis model (3). An enriched population of inflammatory Mφ were elicited with thioglycollate, and mice were then treated with a bolus intraperitoneal injection of LXA₄ (1 or 2 μg for 15 min) before instillation of CMO-labeled apoptotic human PMN. It was necessary to use this approach given the difficulties in assessing endogenous gen-

Figure 4. LX rapidly stimulate Mφ phagocytosis of apoptotic PMN in vivo. BALB/C adult mice were treated with thioglycolate (3%) for 5 d before intraperitoneal administration of LX or vehicle (15 min) before instillation of aged human CMO-labeled PMN. Cytospin preparations of peritoneal lavages were prepared, and ingested PMN was quantified by fluorescent microscopy. In each experiment, a minimum of 400 cells was counted and the number of Mφ containing one or more apoptotic PMN was expressed as a percentage of the total number of Mφ. (A) Upper panel, CMO-labeled aged PMN; middle panel, auto-fluorescent lavaged macrophages; lower panel, LXA₄-stimulated Mφ phagocytosis of apoptotic PMN in vivo; the thin arrows point to ingested PMN. (B) LXA₄ stimulates phagocytosis in vivo. A 250-μl bolus of vehicle or LXA₄ (1 μg and 2 μg) was administered intraperitoneally for 15 min before a 30-min PMN coincubation. Phagocytosis was assessed as described above. Data are mean % phagocytosis ± SEM (n = 3); *P < 0.005 versus control. (C) LXA₄ significantly stimulates phagocytosis after 15-min and 30-min coincubation with excess apoptotic PMN. Mice were treated with 1 μg LXA₄ for 15 min before either a 15-min or a 30-min coincubation with apoptotic PMN in vivo. Phagocytosis was assayed as described above. Data are expressed as mean % phagocytosis ± SEM (n = 3); *P < 0.005; **P < 0.0005 versus control.
eration and processing of apoptotic cells during acute inflammation. Thirty minutes after the instillation of apoptotic PMN intraperitoneally, lavaged cells were assayed by fluorescence microscopy. Figure 4A shows CMO-labeled apoptotic PMN, autofluorescence lavaged macrophages, and lavaged macrophages from LX-treated mice. LXA₄ (1 μg and 2 μg) stimulated a 1.8-fold and a 2.2-fold increase in phagocytosis, respectively, as demonstrated in Figure 4B. Significant enhancement of phagocytosis was also seen in LXA₄-treated mice when lavages were performed 15 min and 30 min after the instillation of apoptotic PMN (Figure 4C). The ability of LXB₄, the 15 epimeric ATL, and stable synthetic LXA₄ analogue to influence clearance of apoptotic PMN at an inflammatory site was also investigated. As seen with LXA₄, LXB₄ (1 μg) promoted phagocytosis in vivo an action shared with the aspirin-triggered epimer 15-epi-LXB₄ (0.5 μg) and 15(R/S)-methyl-LXA₄ (0.5 μg), which secured a twofold increase in phagocytosis (Figure 5). In aggregate, these data provide the first demonstration that native LX, ATL, and LX stable analogues promote clearance of apoptotic PMN in vivo and support their role as rapidly acting endogenous pro-resolution agents in inflammation.

Discussion

Here we demonstrate that LX (LXA₄, LXB₄, ATL, and stable synthetic LX analogues) and the peptide mimetics (MHC bp and MMK-1) can promote non-phlogistic phagocytosis of apoptotic PMN in vitro that is coupled to TGF-β₁ release, insensitive to PSR antisera, and involves the CD36-α₋β₃ complex. We demonstrate that LX stimulate phagocytosis of exogenously administered excess apoptotic PMN in a murine model in vivo, suggesting that LX rapidly promote the clearance of apoptotic leukocytes within an inflammatory milieu. Non-phlogistic clearance of apoptotic leukocytes by phagocytosis is an important determinant of the resolution of inflammation. The benefits of such phagocytosis are not restricted merely to clearance of potentially lytic cells; such phagocytosis may be coupled to the active suppression of inflammation. Ultrastructural studies have demonstrated apoptotic uptake by Mφ in glomerulonephritis (31), whereas the persistence of apoptotic bodies in the glomeruli of C1q-deficient mice is indicative of impaired clearance (32).

To investigate the mechanism whereby LX-stimulated Mφ phagocytosis of apoptotic PMN, we have used in vitro models: LX-stimulated human Mφ and cytokine-primed rat BMDM. In preliminary experiments, we investigated whether LX might stimulate phagocytosis of targets other than apoptotic PMN, namely latex beads and apoptotic lymphocytes. However, in our experiments the level of phagocytosis observed basally in vivo was essentially uncommitted phagocytes that can develop phenotypically distinct properties after cytokine programming, resulting in the development of inflammatory (TGF-β), cytotoxic (IFN-γ/TNF-α), and reparative (TNF-α) phenotypes (30). Exposure of TNF-α-primed Mφ to LX further enhances their ability to phagocytose apoptotic PMN, whereas LX rescues the compromised phagocytic activity of TNF-α- and IFN-γ-primed Mφ. These data suggest that LX may stimulate PMN clearance by Mφ within the complex multi-mediator milieu that typifies inflammation in vivo.

To identify the signaling molecules involved in LX-stimulated phagocytosis, we have employed modulators of pathways implicated in LX signaling (15,33) and/or phagocytosis (reviewed in reference 34). We demonstrate a role for PKC and PI 3-kinase in LX-stimulated phagocytosis. It is likely that LXA₄ and LXB₄ stimulate phagocytosis through novel PKC isoforms (35) on the basis of the contrasting actions of the two LX on calcium mobilization (36). The 3'-phosphorylated substrates of PI 3-kinase are important regulators of several downstream components of phagocytosis, including Rho activation, a consequence of LX stimulation of Mφ (37).

A modulatory role for cAMP is suggested by our observations that both LXA₄ and LXB₄-stimulated phagocytosis was inhibited by cell-permeable 8 Br-cAMP and mimicked by a PKA inhibitor. In this context, it is noteworthy that phosphorylation of CD36 by PKA may reduce its ability to bind apoptotic cells (16,38); furthermore, phagocytosis is dependent on cytoskeletal rearrangements, which are regulated by monomeric GTPases, including Rho (39), and Rho activation is negatively regulated by PKA (40).

The cell surface molecules involved in Mφ-apoptotic cell recognition include lectins, integrins, such as Mφ α₋β₃ that co-operate with CD36, PSR, scavenger receptors, and CD14 and specific bridging molecules, including thrombospordin and C1q (1). The ligand for Mφ receptors on apoptotic cells are less well documented; activation of a phospholipid scramblase in apoptotic cells results in exposure of phosphatidylserine (41), a ligand for the recently described Mφ PSR (23). PS-

![Figure 5. LX-stimulated Mφ phagocytosis of apoptotic PMN in vivo.](image-url)
dependent phagocytosis of apoptotic leukocytes by mouse thioglycollate-elicited peritoneal Mφ has previously been shown, whereas quiescent Mφ were reported to utilize an αβ3-dependent mechanism (1). Recent data indicate that phagocytosis by either activated or quiescent Mφ is PSR-dependent (23,29). Here we report that in monocyte-derived Mφ LX-stimulated phagocytosis required the αβ3-CD36 complex but was independent of the PSR. This conclusion is based on sensitivity to anti-αβ3 and CD36 antisera and insensitivity to PSR antisera. Further evidence of PSR independence are provided from β1,3-glucan–treated Mφ. This maneuver subverts the αβ3–CD36 system to a PSR-dependent mechanism (25). LX-dependent phagocytosis was not observed in β1,3-glucan–treated Mφ (importantly, the previously documented effects of stimulation of Mφ with PSR antisera and with β1,3-glucan on stimulating TGF-β release (25,28,42) were observed). Therefore, LX-stimulated phagocytosis of apoptotic cells is not necessarily dependent on the PSR. An analogous situation has recently been described by Cocco and Ucker (43) and for dexamethasone-augmented phagocytosis (44). A role for PS in LX-stimulated phagocytosis is not excluded as PS may bind to alternative receptors, e.g., a ligand for the Axl/Tyro3 Mer family of receptor tyrosine kinase growth arrest specific gene 6 (GAS 6) mediates PS binding to Mφ (45), and PS facilitates CD36-TSP-αβ3-mediated uptake of apoptotic cells (25).

The LX4 receptor (ALXR) (also known as the FPRL-1 receptor) is a G-protein–coupled receptor (46) expressed in diverse cell types (4). LXB4 does not compete with [3H]LX4 for binding to the ALXR in PMN (47). In addition to binding LX4 and its analogues, the ALXR has recently been shown to bind specific peptides (19,27), the acute phase protein serum amyloid A (SAA) (48), prion protein (49), and β amyloid (50). More recently, an additional receptor for peptide agonists has been described, namely FPRL-2. FPRL-2 is expressed on monocyte/macrophages but not PMN (51). LX and its analogues act via the ALXR to inhibit PMN chemotaxis. In contrast to the inhibitory effects of LX on PMN function, LX have been reported to activate monocyte chemotaxis, and this effect is shared with the ALXR peptide ligands (18). This contrasting data obtained from peptide and LX ligands in monocytes and PMN suggests involvement of the FPRL-2 receptor (19,27,51). It was of interest to investigate whether peptide agonists of ALXR could mimic the effects of LX on phagocytosis of apoptotic PMN. The peptides investigated were an MHC bp derived from mitochondrial NADPH dehydrogenase and a synthetic rogue peptide (MMK-1) (19,27). Exposure of Mφ to either peptide stimulated phagocytosis to levels comparable to those seen with LX. Interestingly, activity was not observed on addition of suboptimal concentrations of LX and peptides together, suggesting a common mechanism. The release of N-formylated peptides from mitochondria of damaged cells is a signal for PMN chemotaxis. On the basis of our data, we propose that MHC bp and MMK-1 peptides might also act to stimulate macrophage phagocytosis of apoptotic cells via the ALXR or the more recently described FPRL-2 (51). We have verified the potential for peptide-activated phagocytic activity in the resolution of inflammation, showing that it is coupled to TGF-β1 release.

Our data demonstrating LX-stimulated phagocytosis of apoptotic PMN in vitro suggest a role for this process in promoting the resolution of inflammation in vivo. Consistent with this hypothesis are the data that demonstrate that LX4 and LXB4 stimulated phagocytosis of human apoptotic PMN in the murine BALB/C model of thioglycollate-induced peritonitis, an established model of inflammation (3,52). Furthermore, the aspirin-triggered 15-epi-LXB4 and the stable LX analogue 15(R/S)-methyl-LX4 (19) also stimulated phagocytosis. The enhanced efficacy and stability of LX analogues in modulation of leukocyte trafficking in models of inflammation and second organ injury after local and systemic administration has led to the proposal that these compounds might have therapeutic potential (17–20). Consistent with this are our data that highlight the attractiveness of LX stable analogues, which are resistant to metabolic inactivation by monocytes/macrophages yet retain the activity of the native LX (17,19,21). Comparable stimulation of phagocytosis was observed with 0.5–µg doses of 15(R/S)-methyl-LX4 and 15-epi-LXB4 to that seen with 2–µg doses of the native LX4. However, it was not feasible to investigate detailed dose-response relationships; therefore, conclusions about relative potency and efficacy in this model cannot be made. In previous reports (19), 10 µg of intravenously administered ATL were found to be effective in modulating leukocyte trafficking; it is obviously difficult to make a direct comparison between the availability via the different routes of administration. As described previously (see above), aspirin acetylation of COX-2 promotes the biosynthesis of epimeric LX (18), which can act locally to downregulate leukocyte activity (18–20) and, as suggested by our data, promote the clearance of apoptotic PMN from an inflammatory site. This may be particularly relevant in inflammation, where COX-2 expression is upregulated (4) and may have clinical implications for the use of COX-2 inhibitors, which leave the 15 lipoxygenase activity of the enzyme intact.

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References


42. McDonald PP, Fadok VA, Bratton D, Henson PM: Transcriptional and translational regulation of inflammatory mediated production by endogenous TGF-β in macrophages that have ingested apoptotic cells. *J Immunol* 163: 6164–6171, 1999


Lipoxins Stimulate Phagocytosis In Vivo

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