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# 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 $LXA_4$ stimulates non-phlogistic phagocytosis of apoptotic polymorphonuclear neutrophils (PMN) by monocyte-derived macrophages (M$\phi$) *in vitro*, suggesting a role for LX as endogenous pro-resolution lipid mediators. It is here reported that $LXA_4$, $LXB_4$, the aspirin-triggered LX (ATL) epimer, 15-epi-$LXB_4$, and a stable synthetic analogue 15(*R/S*)-methyl-$LXA_4$ stimulate phagocytosis of exogenously administered excess apoptotic PMN by macrophages (M$\phi$) *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 $LXA_4$, $LXB_4$, 15(*R/S*)-methyl-$LXA_4$, and 15-epi-$LXB_4$ of 2.5 to 10 $\mu$g/kg. Non-phlogistic LX-stimulated phagocytosis by M$\phi$ was sensitive to inhibition of PKC and PI 3-kinase and associated with increased production of transforming growth factor–$\beta_1$ (TGF-$\beta_1$). LX-stimulated phagocytosis was not inhibited by phosphatidylserine receptor (PSR) antisera and was abolished by prior exposure of M$\phi$ to $\beta$1,3-glucan, suggesting a novel M$\phi$-PMN recognition mechanism. Interestingly, the recently described peptide agonists of the $LXA_4$ receptor (MYFINITL and LESIFRSLLFRVM) stimulated phagocytosis through a process associated with increased TGF-$\beta_1$ release. These data provide the first demonstration that $LXA_4$, $LXB_4$, ATL, and LX stable analogues rapidly promote M$\phi$ 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.

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 (M$\phi$) 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 M$\phi$ has been implicated in the pathogenesis of chronic inflammatory conditions, including glomerulonehritis (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-

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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). $LXA_4$ and $LXB_4$ inhibit $LTB_4$-induced PMN chemotaxis and PMN-endothelial cell adhesion supported by CD11/CD18 integrins (7). $LXA_4$ and $LXB_4$ also attenuate $LTC_4$- and $LTD_4$-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 $A_4$ [(5*S*,6*R*,15*S*)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid] and its positional isomer lipoxin $B_4$ [(5*S*,14*R*,15*S*)-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 $\omega$-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 15*R* lipoxygenase. In this context, 15(*R*)-HETE is produced and can then be metabolized to 15 epimers of $LXA_4$ and $LXB_4$ during cell-cell interactions (18). These aspirin-triggered lipoxins (ATL) also possess antiinflammatory activity (19,20).

Here we demonstrate that LX, ATL, 15-epi-$LXB_4$, and the stable synthetic LX analogue 15(*R/S*)-methyl-$LXA_4$ promote M$\phi$ 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–$\beta_1$ (TGF-$\beta_1$) 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 lipoxygenase-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). $LXA_4$ and $LXB_4$ were obtained from Cascade Biologicals (Berkshire, UK). 15(*R/S*)-methyl-$LXA_4$, 16-phenoxy-$LXA_4$ and the aspirin-triggered 15-epi-$LXB_4$ were prepared by total organic synthesis (21). CM-orange (CMO, cell tracker orange) was from Molecular Probes (Oregon, US). Rat cytokines tumor necrosis factor–$\alpha$ (TNF-$\alpha$), TGF-$\beta$, interferon-$\gamma$ (IFN-$\gamma$), and TGF-$\beta_1$ and MCP-1 human enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (Oxon, UK). The anti-CD36 monoclonal antibody SM$\phi$ (IgM) was provided by Dr. Nancy Hogg (ICRF, London) (22). Anti-$\alpha_v\beta_3$ mouse monoclonal antibody (23C6) was purchased from Serotec (Oxford, UK). FITC-conjugated anti-CD36 (FA6–152) and anti-$\alpha_v\beta_3$ (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 Calbiochem (Nottingham, UK). $\beta$1,3-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). M$\phi$ 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.

### *M$\phi$ Phagocytosis of Apoptotic PMN In Vitro*

M$\phi$ were treated with reagents as indicated and washed with RPMI 1640 before coincubation with aged PMN in 24-well tissue culture plates ($4 \times 10^6$ 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). M$\phi$ were routinely negative for peroxidase staining. For each experiment, the number of M$\phi$ containing one or more PMN in at least five fields (minimum of 400 cells) was expressed as a percentage of the total number of M$\phi$ 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 $500 \times g$ 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 tetraoxide 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-$\mu$m sections were cut with a glass knife and stained with toluene 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 $\mu$m.

### *Inhibitor and Antibody Studies*

M$\phi$ 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 $\mu$M) (16), the PI 3-kinase inhibitor LY 294002 (10 $\mu$M) (15), the PKC inhibitor GF 109203X (10 $\mu$M) (15), the MAP kinase inhibitor PD 98059 (10 $\mu$M) (15), or appropriate vehicles for 15 min at 37°C before stimulation with $LXA_4$ or $LXB_4$ (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 M$\phi$ to anti-CD51/61 mAb (20 $\mu$g/ml) and anti-CD36 mAb (SM$\phi$) ascitic fluid (22) (125 $\mu$l/ml) on ice for 20 min. M$\phi$ were then treated with LX (1nM; 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), M$\phi$ were treated with PSR antisera (200 $\mu$g/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 M$\phi$*

Treatment of M$\phi$ with $\beta$1,3-glucan confers PSR dependence on M$\phi$ phagocytosis of apoptotic PMN (25). On day 5, human M$\phi$ were treated with $\beta$1,3-glucan (25 $\mu$g/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 M$\phi$ (BMDM) were isolated by standard procedures (26). Briefly, bone marrow cells from the dissected femurs of male Sprague-Dawley rats were flushed 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 d, cells were plated into 24-well plates at a concentration of $5 \times 10^5$ cells/well and rested for 24 h in M-CSF–free medium. Cells were stimulated with murine TNF-$\alpha$ (10 ng/ml for 24 h), IFN-$\gamma$/TNF-$\alpha$ (10 ng/ml for 4 h and 10 ng/ml for 20 h), or TGF-$\beta$ (5 ng/ml for 24 h) before $LXA_4$ treatment (1 nM for 15 min). Phagocytosis of aged human PMN was assayed as above.

### *Assay of TGF-$\beta_1$ and MCP-1 Release from M$\phi$*

TGF-$\beta_1$ and MCP-1 were assayed in supernatants from co-cultures of PMN-M$\phi$ by ELISA according to the manufacturer's instruction (R&D systems).

### *Assessment of LX-Stimulated M$\phi$ 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 M$\phi$ 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. $LXA_4$, $LXB_4$, 15(*R/S*)-methyl-$LXA_4$, 16-phenoxy-$LXA_4$, and 15-epi-$LXB_4$ were administered intraperitoneally 15 min before instillation of a bolus of labeled, apoptotic PMN (approximate 3:1 ratio of PMN to M$\phi$) (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 M$\phi$ containing ingested CMO-positive, apoptotic PMN was determined by fluorescence microscopy and expressed relative to total number of M$\phi$ 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 M$\phi$ phagocytosis of apoptotic PMN as demonstrated by electron microscopy. As previously reported for $LXA_4$ *in vitro* (16), $LXB_4$ 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. $LXA_4$ and $LXB_4$ demonstrated similar dose-response relationships with an $EC_{50}$ of $0.5 \times 10^{-9}$ M and a maximum stimulation observed at 1 nM (Figure 1B). Interestingly, additivity was not observed with simultaneous exposure of M$\phi$ to $LXA_4$ and $LXB_4$ (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 epi-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-$LXA_4$ also stimulated macrophage phagocytosis (Figure 1B). Recent data indicate that, in addition to $LXA_4$ 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 M$\phi$ phagocytosis of apoptotic PMN. Treatment of M$\phi$ 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 $LXA_4$ (Figure 1C) when used at a suboptimal concentration (0.5 nM)

In contrast to phagocytosis of opsonized particles, nonphlogistic M$\phi$ phagocytosis of apoptotic PMN may contribute to the resolution of inflammation, being characteristically coupled with TGF-$\beta_1$ 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, M$\phi$ were treated with $LXA_4$, MHC bp, and MMK-1 (1 nM for 15 min) before coincubation with apoptotic PMN, and TGF-$\beta_1$ and

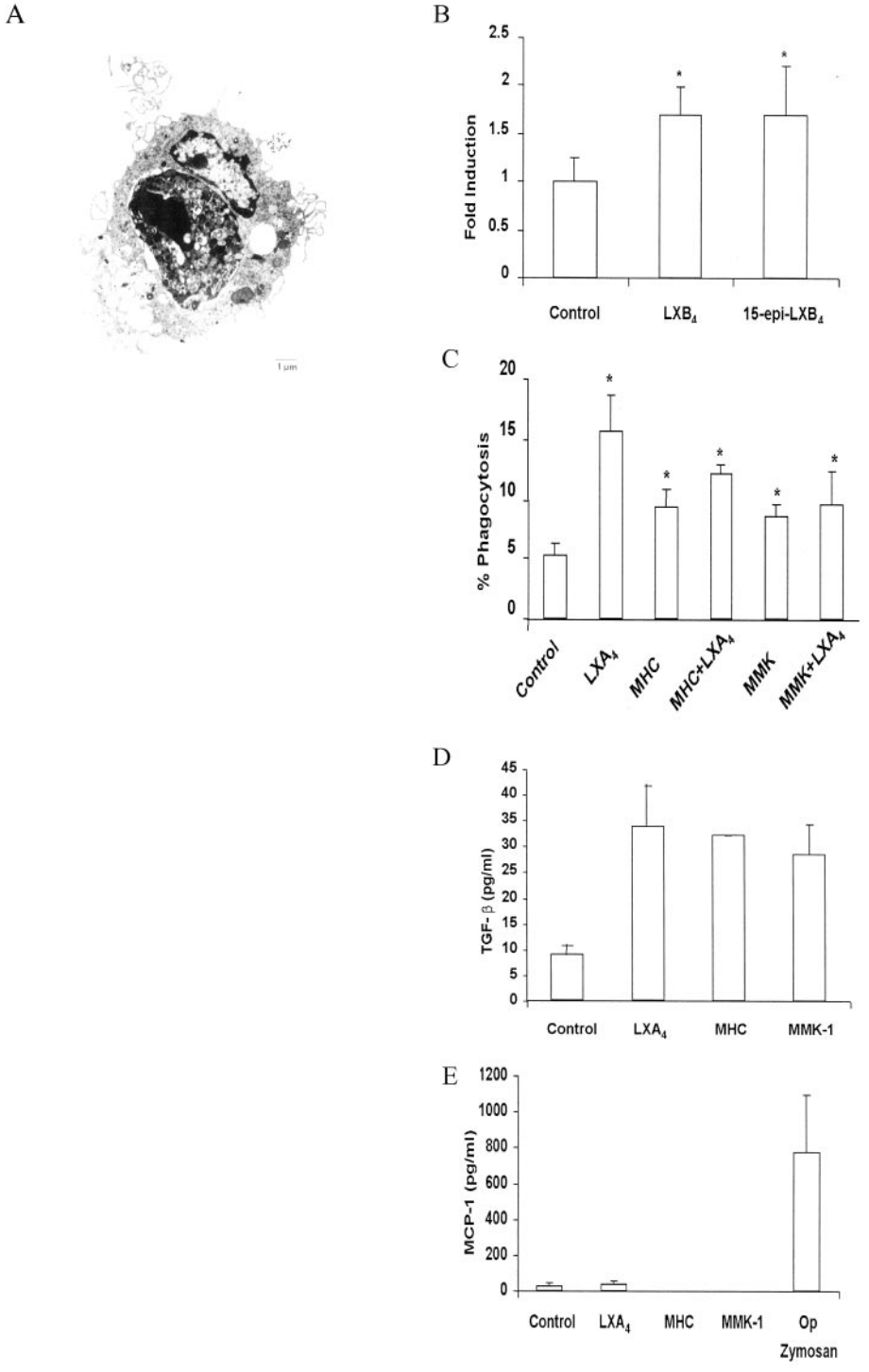


*Figure 1.* Lipoxins (LX), aspirin-triggered LX (ATL), and peptide agonists promote non-phlogistic macrophage (M$\phi$) phagocytosis of apoptotic polymorphonuclear neutrophils (PMN). (A) M$\phi$ were treated with $LXA_4$ (1nM for 15 mins) before coincubation with aged PMN for 30 min. Electron microscopy was performed as described in Materials and Methods. (B) $LXB_4$ and the aspirin-triggered 15-epi-$LXB_4$ stimulate M$\phi$ phagocytosis. M$\phi$ were treated with $LXB_4$ (1 nM) or 15-epi-$LXB_4$ ($1 \times 10^{-11}$ M) for 15 min before coincubation with aged PMN for 30 min. Data are fold induction $\pm$ SEM ($n = 4$); *$P < 0.05$ *versus* control. (C) Peptide agonists of $LXA_4$ receptor (ALXR) stimulate M$\phi$ phagocytosis. M$\phi$ were treated with MHC bp or MMK-1 synthetic peptide (1 nM for 15 min) alone or in combination with $LXA_4$ (1 nM) for 15 min before coincubation with aged PMN for 30 min. Data are mean $\pm$ SEM ($n = 4$); *$P < 0.05$ *versus* control. (D) $LXA_4$ and peptide agonists stimulate the release of transforming growth factor–$\beta_1$ (TGF-$\beta_1$). M$\phi$ were treated with $LXA_4$, 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-$\beta_1$ production was measured by enzyme-linked immunosorbent assay (ELISA). Data are expressed as mean TGF-$\beta_1$ production (pg/ml) $\pm$ SD. (E) $LXA_4$ and peptide agonists do not provoke release of MCP-1. M$\phi$ were treated with $LXA_4$, 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) $\pm$ SD.

MCP-1 production was assayed by ELISA. $LXA_4$ treatment induced a sixfold increase in TGF-$\beta_1$ production (Figure 1D). Time course analysis of TGF-$\beta_1$ release after LX-stimulated M$\phi$ ingestion of apoptotic PMN showed an increase from 30 min to 18 h (data not shown). Interestingly, the peptides MHC bp and MMK-1 also evoked an increase in TGF-$\beta_1$ production (Figure 1D). In contrast, $LXA_4$, MHC bp, and MMK-1 treatment of M$\phi$ did not elicit MCP-1 release after 30 min or 18 h M$\phi$-PMN coincubation (Figure 1E).

### *LX-Stimulated Phagocytosis: The Role of cAMP, PKC, PI 3-kinase, and MAP Kinase*

The signal transduction pathways mediating M$\phi$ phagocytosis of apoptotic PMN are poorly understood. A role for cyclicAMP in this process has been demonstrated. Consistent with previous findings for $LXA_4$ (16), $LXB_4$-stimulated phagocytosis was inhibited by the cell-permeable analogue 8-Br-cAMP and mimicked by the PKA inhibitor Rp-cAMP (vehicle, 34.5 ± 5.35; $LXB_4$, 64.5 ± 7.5;* 8-Br-cAMP, 21.6 ± 3.2; 8-Br-cAMP + $LXB_4$, 17.63 ± 2.7; Rp-cAMP, 50.0 ± 2.98;* Rp-cAMP + $LXB_4$, 68.0 ± 5.3;* data are % phagocytosis ± SEM; $n = 4$ *$P < 0.05$ *versus* control).

Given the important role of PI 3-kinase, PKC and erk as modulators of M$\phi$ cytoskeletal and/or adhesive events critical to phagocytic activity (1), we explored their role in LX-stimulated phagocytosis of apoptotic PMN. Previous exposure of M$\phi$ to the PKC inhibitor GF 109203X blunted LX-triggered phagocytosis (Table 1). The PI 3-kinase inhibitor LY 294002 also reduced LX-stimulated phagocytosis. In contrast, the MAP kinase inhibitor PD 98059 was without effect on LX-stimulated phagocytosis (control, 11.5 ± 3.4; $LXB_4$, 21.5 ± 5.3; PD, 11.3 ± 3.6; PD + $LXB_4$, 22.1 ± 4.6; % phagocytosis ± SEM). Interestingly, the serine-threonine phosphatase inhibitor okadaic acid inhibited LX-stimulated phagocytosis, suggesting a modulatory role for phosphatases in the resolution process (Table 1).

### *Enhancement of M$\phi$ Phagocytosis of Apoptotic PMN In Vitro: A PSR-Independent Mechanism*

In the present study, $LXB_4$-stimulated phagocytosis, similar to the $LXA_4$-induced response (16), was inhibited by mAbs against M$\phi$ CD36 and $\alpha_v\beta_3$ integrins (control, 14.5 ± 3.25; $LXB_4$, 28.6 ± 6.16;* anti-CD36 mAb, 13.93 ± 3.49; anti-CD36 mAb + $LXB_4$, 14.60 ± 4.44; anti-CD51/61 mAb, 14.10 ± 1.83; anti-CD51/61 mAb + $LXB_4$, 12.58 ± 2.36; % phagocytosis ± SEM; $n = 3$; *$P < 0.05$ *versus* control). The M$\phi$ PSR is an important molecule in M$\phi$ recognition of apoptotic cells in many systems (23). Here we evaluated the role of the PSR in $LXA_4$ and $LXB_4$-stimulated phagocytosis using PSR antisera. PSR antisera (200 $\mu$g/ml) did not inhibit LX-stimulated phagocytosis (Figure 2A). Exposure of M$\phi$ to $\beta$1,3-glucan renders M$\phi$-PMN interaction PSR-dependent (25). Here we report that treatment of M$\phi$ with 25 $\mu$g/ml $\beta$1,3-glucan (48 h) abolished both $LXA_4$ and $LXB_4$-stimulated phagocytosis (Figure 2B). To verify that both the PSR antisera and $\beta$1,3-glucan were functional in our system and capable of eliciting previously reported effects (23,25,28,29), we measured TGF-$\beta_1$ release after 18-h treatment with PSR antisera or $\beta$1,3-glucan; both agents induced significant TGF-$\beta_1$ production (Figure 2C).

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

| | % Increase in Phagocytosis ± SEM |
|---|---|
| $LXB_4$ | 84.8 ± 2.0 |
| GF | 4.0 ± 0.9[b] |
| GF 109203X + $LXB_4$ | 4.0 ± 1.8[b] |
| $LXB_4$ | 141.4 ± 7.1 |
| LY 294002 | 31.0 ± 3.8[b] |
| LY 294002 + $LXB_4$ | 68.9 ± 4.0[b] |
| $LXB_4$ | 87.5 ± 11.6 |
| Okadaic acid | 16.5 ± 10.4[b] |
| Okadaic acid + $LXB_4$ | 5.8 ± 7.2[b] |

[a] M$\phi$ were treated with GF 109203X (20 $\mu$M), LY 294002 (10 $\mu$M), and Okadaic acid (100 nM) for 15 min at 37°C before stimulation with $LXB_4$ (1 nM for 15 mins at 37°C). Data are % increase in phagocytosis *versus* control ± SEM ($n = 5$).
[b] $P < 0.05$ *versus* control.

### *LX Reprograms BMDM Phenotype*

BMDM can be programmed to develop distinct phenotypes in response to exposure to specific cytokines, including TGF-$\beta$, IFN-$\gamma$/TNF-$\alpha$, and TNF-$\alpha$ (30). IFN-$\gamma$/TNF-$\alpha$ stimulate BMDM to adopt a cytotoxic phenotype characterized by sustained NO production and a diminished phagocytic capacity (30). Exposure of rat BMDM to TNF-$\alpha$ 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. $LXA_4$ and stable LX analogues significantly stimulated naïve rat BMDM to phagocytose apoptotic PMN (Figure 3A).

After programming by cytokine-programmed cohorts (Figure 3, B and C): TNF-$\alpha$ treatment (10 ng/ml for 24 h) stimulated a significant increase in phagocytosis that was further enhanced by stimulation with $LXA_4$ ($1 \times 10^{-9}$ M for 15 min), 15(*R/S*)-methyl-$LXA_4$ ($1 \times 10^{-11}$ M for 15 min), or 16-phenoxy-$LXA_4$ ($1 \times 10^{-11}$ M for 15 min) (Figure 3B). Exposure to IFN-$\gamma$/TNF-$\alpha$ (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 $LXA_4$ ($1 \times 10^{-9}$ M for 15 min), 15(*R/S*)-methyl-$LXA_4$ ($1 \times 10^{-11}$ M for 15 min), or 16-phenoxy-$LXA_4$ ($1 \times 10^{-11}$ M for 15 min) rescued this phenotype and a significant increase in phagocytosis was observed comparable to naïve BMDM (Figure 3C).

### *LX Stimulate M$\phi$ Phagocytosis of Exogenously Administered Apoptotic PMN In Vivo*

To determine if LX stimulate M$\phi$ phagocytosis of apoptotic PMN *in vivo*, we employed the mouse (BALB/C) thioglycol-

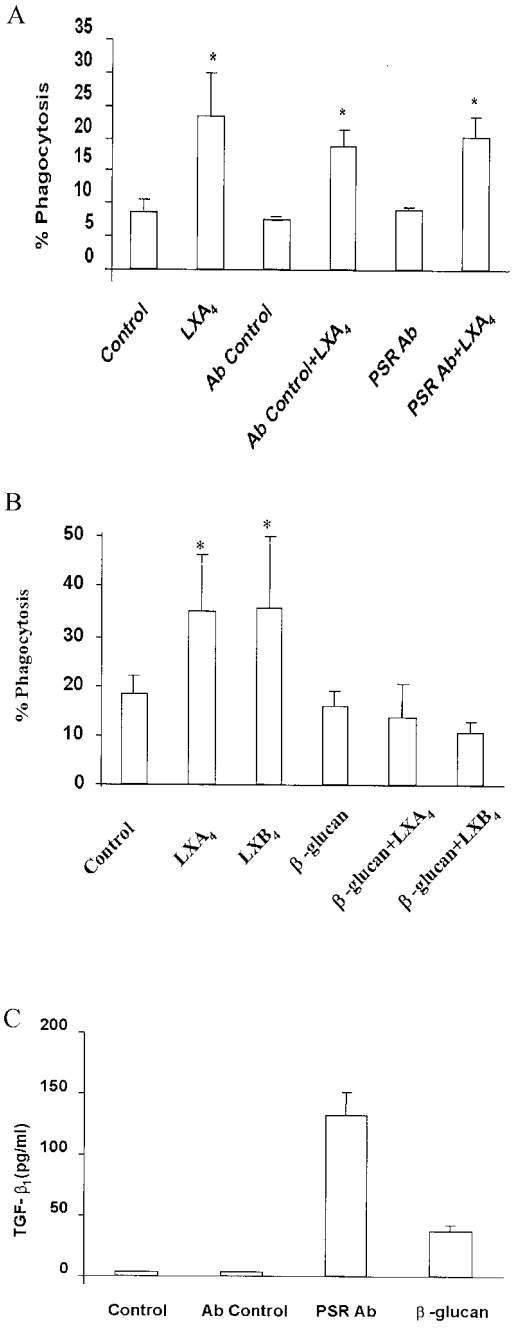


*Figure 2.* LX-stimulated phagocytosis is phosphatidylserine receptor–independent. (A) Phosphatidylserine receptor (PSR) antisera did not block LX-stimulated phagocytosis. M$\phi$ were treated with PSR antisera (200 $\mu$g/ml) or isotype control Ab on ice for 30 min before treatment with $LXA_4$ (1 nM for 15 mins at 37°C) and subsequent coincubation with apoptotic PMN. Data are mean % phagocytosis ± SEM ($n = 3$); *$P < 0.05$. (B) $\beta$1,3-glucan treatment abolishes sensitivity of M$\phi$ to LX-stimulated phagocytosis. M$\phi$ were treated with 25 $\mu$g/ml $\beta$1,3-glucan for 48 h before treatment with $LXA_4$ and $LXB_4$ (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 $\beta$1,3-glucan treatment of M$\phi$ elicits TGF-$\beta_1$ release. TGF-$\beta_1$ release from M$\phi$ treated with control, PSR Ab (200 $\mu$g/ml), isotype control Ab, and $\beta$1,3-glucan (25 $\mu$g/ml) for 18 h at 37°C was determined by ELISA. Data are expressed as mean cytokine production ± SD.

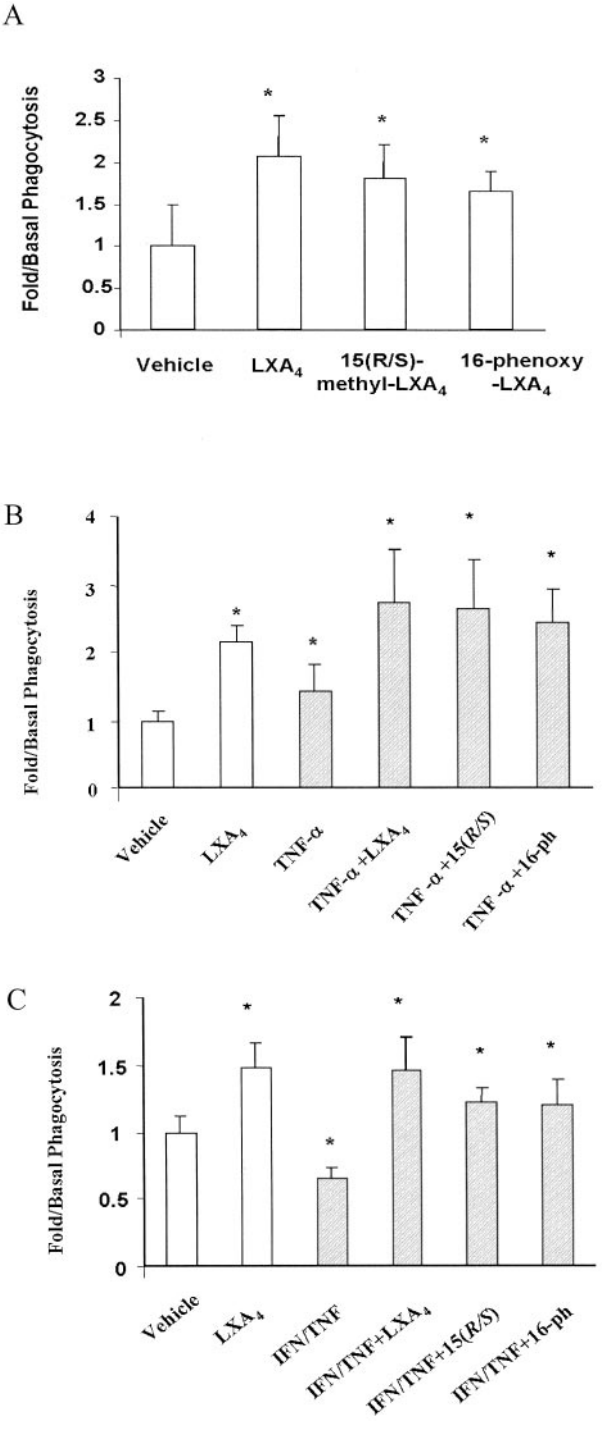


*Figure 3.* LX stimulate phagocytosis of bone marrow–derived M$\phi$ (BMDM) under basal conditions and after cytokine programming. (A) BMDM were treated with $LXA_4$ (1 nM for 24 h), 15(*R/S*)-methyl-$LXA_4$, and 16-phenoxy-$LXA_4$ ($1 \times 10^{-11}$ M for 24 h). BMDM were treated with (B) tumor necrosis factor–$\alpha$ (TNF-$\alpha$) (10 ng/ml for 24 h) or (C) interferon-$\gamma$ (IFN-$\gamma$)/TNF-$\alpha$ (10 ng/ml for 4 h and 10 ng/ml for 20 h) before treatment with $LXA_4$ (1 nM for 15 mins), 15(*R/S*)-methyl-$LXA_4$ or 16-phenoxy-$LXA_4$ ($1 \times 10^{-11}$ 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 M$\phi$ that had ingested one or more PMN. Results are expressed as fold/basal phagocytosis ± SEM ($n = 4$); *$P < 0.05$ *versus* control.

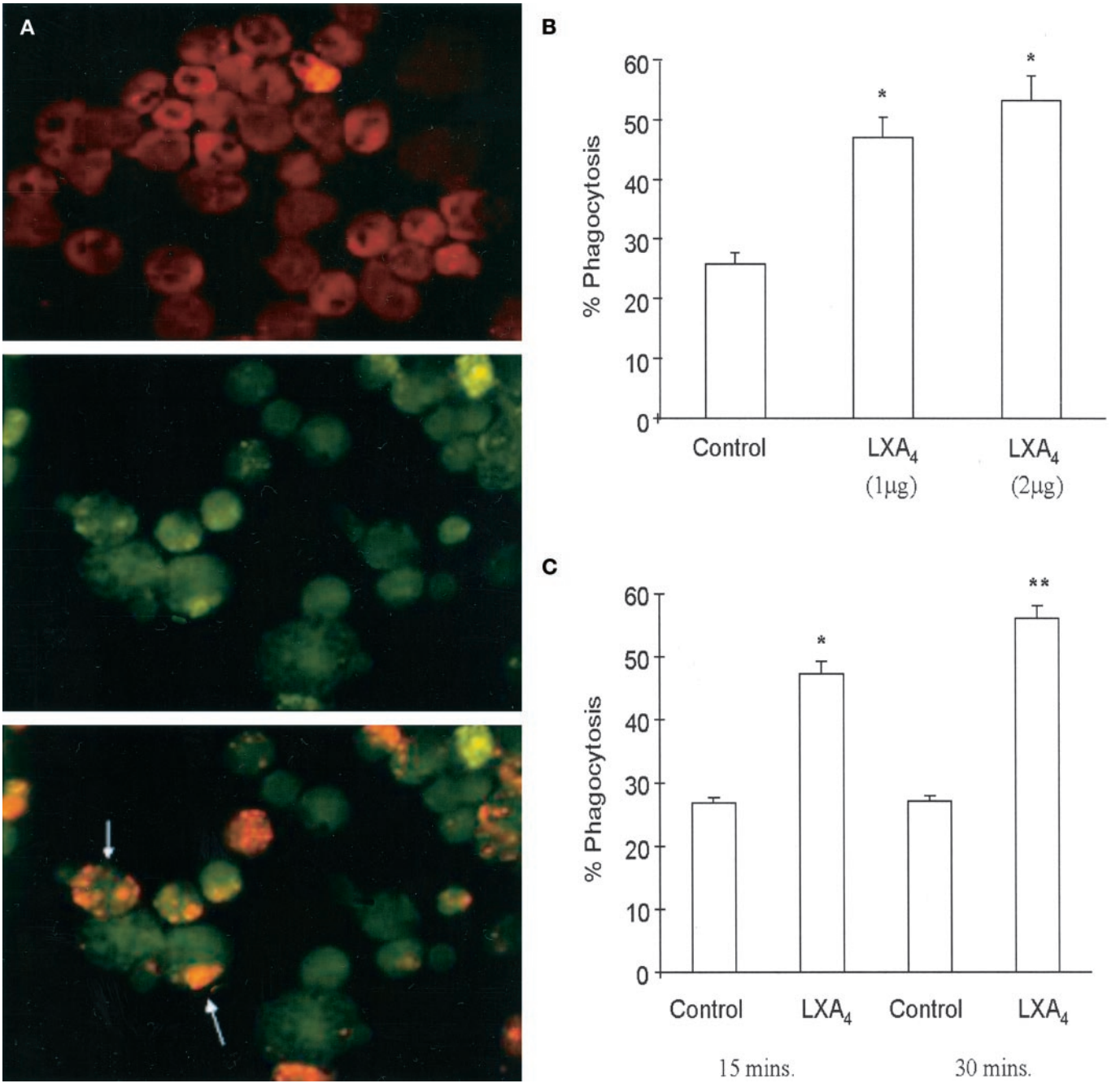


*Figure 4.* LX rapidly stimulate M$\phi$ phagocytosis of apoptotic PMN *in vivo*. BALB/C adult mice were treated with thioglycolate (3%) for 5 d before intraperitoneal administration of LX or vehicle (15 min) before instillation of aged human CMO-labeled PMN. Cytospin preparations of peritoneal lavages were prepared, and ingested PMN was quantified by fluorescent microscopy. In each experiment, a minimum of 400 cells was counted and the number of M$\phi$ containing one or more apoptotic PMN was expressed as a percentage of the total number of M$\phi$. (A) Upper panel, CMO-labeled aged PMN; middle panel, auto-fluorescent lavaged macrophages; lower panel, $LXA_4$-stimulated M$\phi$ phagocytosis of apoptotic PMN *in vivo*; the thin arrows point to ingested PMN. (B) $LXA_4$ stimulates phagocytosis *in vivo*. A 250-$\mu$l bolus of vehicle or $LXA_4$ (1 $\mu$g and 2 $\mu$g) was administered intraperitoneally for 15 min before a 30-min PMN coincubation. Phagocytosis was assessed as described above. Data are mean % phagocytosis $\pm$ SEM ($n = 3$); *$P < 0.005$ *versus* control. (C) $LXA_4$ significantly stimulates phagocytosis after 15-min and 30-min coincubation with excess apoptotic PMN. Mice were treated with 1 $\mu$g $LXA_4$ 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 $\pm$ SEM ($n = 3$); *$P < 0.005$; **$P < 0.0005$ *versus* control.

late-elicited peritonitis model (3). An enriched population of inflammatory M$\phi$ were elicited with thioglycollate, and mice were then treated with a bolus intraperitoneal injection of $LXA_4$ (1 or 2 $\mu$g 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-

eration and processing of apoptotic cells during acute inflammation. Thiry 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. $LXA_4$ (1 $\mu$g and 2 $\mu$g) 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 $LXA_4$-treated mice when lavages were performed 15 min and 30 min after the instillation of apoptotic PMN (Figure 4C). The ability of $LXB_4$, the 15 epimeric ATL, and stable synthetic $LXA_4$ analogue to influence clearance of apoptotic PMN at an inflammatory site was also investigated. As seen with $LXA_4$, $LXB_4$ (1 $\mu$g) promoted phagocytosis *in vivo* an action shared with the aspirin-triggered epimer 15-epi-$LXB_4$ (0.5 $\mu$g) and 15(*R/S*)-methyl-$LXA_4$ (0.5 $\mu$g), 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 ($LXA_4$, $LXB_4$, 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-$\beta_1$ release, insensitive to PSR antisera, and involves the CD36-$\alpha_{v\beta3}$ 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 M$\phi$ in glomerulonephritis (31), whereas the persistence of apoptotic bodies in the glomeruli of C1q-deficient mice is indicative of impaired clearance (32).

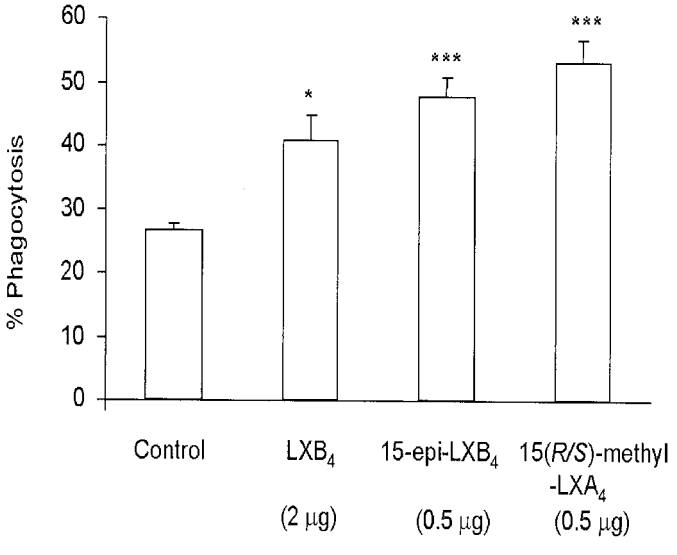


*Figure 5.* LX-stimulated M$\phi$ phagocytosis of apoptotic PMN *in vivo*. An action shared with the ATL and the LX stable analogue. A 250-$\mu$l bolus of control, $LXB_4$ (1 $\mu$g,), 15(*R/S*)-methyl-$LXA_4$ (0.5 $\mu$g), and 15-epi-$LXB_4$ (0.5 $\mu$g) was given intraperitoneally for 15 min before a 30-min PMN coincubation. Phagocytosis was determined as described in Figure 4. Data are expressed as mean % phagocytosis $\pm$ SEM ($n = 3$ to 5); $*P < 0.05$; $***P < 0.0005$ *versus* control.

To investigate the mechanism whereby LX-stimulated M$\phi$ phagocytosis of apoptotic PMN, we have used *in vitro* models: LX-stimulated human M$\phi$ 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 with such materials is extremely high, and it was not possible to observe an incremental increase in response to LX.

BMDM are essentially uncommitted phagocytes that can develop phenotypically distinct properties after cytokine programming, resulting in the development of inflammatory (TGF-$\beta$), cytotoxic (IFN-$\gamma$/TNF-$\alpha$), and reparative (TNF-$\alpha$) phenotypes (30). Exposure of TNF-$\alpha$–programmed M$\phi$ to LX further enhances their ability to phagocytose apoptotic PMN, whereas LX rescues the compromised phagocytic activity of TNF-$\alpha$– and IFN-$\gamma$–primed M$\phi$. These data suggest that LX may stimulate PMN clearance by M$\phi$ 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 $LXA_4$ and $LXB_4$ 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 M$\phi$ (37).

A modulatory role for cAMP is suggested by our observations that both $LXA_4$- (16) and $LXB_4$-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 M$\phi$-apoptoticyte recognition include lectins, integrins, such as M$\phi$ $\alpha_v\beta_3$ that co-operate with CD36, PSR, scavenger receptors, and CD14 and specific bridging molecules, including thrombospondin and C1q (1). The ligand for M$\phi$ 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 M$\phi$ PSR (23). PS-

dependent phagocytosis of apoptotic leukocytes by mouse thioglycollate-elicited peritoneal M$\phi$ has previously been shown, whereas quiescent M$\phi$ were reported to utilize an $\alpha_{v\beta3}$-dependent mechanism (1). Recent data indicate that phagocytosis by either activated or quiescent M$\phi$ is PSR-dependent (23,29). Here we report that in monocyte-derived M$\phi$ LX-stimulated phagocytosis required the $\alpha_{v\beta3}$-CD36 complex but was independent of the PSR. This conclusion is based on sensitivity to anti-$\alpha_v\beta_3$ and CD36 antisera and insensitivity to PSR antisera. Further evidence of PSR independence are provided from $\beta$1,3-glucan–treated M$\phi$. This maneuver subverts the $\alpha_{v\beta3}$-CD36 system to a PS-dependent mechanism (25). LX-dependent phagocytosis was not observed in $\beta$1,3-glucan–treated M$\phi$ (importantly, the previously documented effects of stimulation of M$\phi$ with PSR antisera and with $\beta$1,3-glucan on stimulating TGF-$\beta$ 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 M$\phi$ (45), and PS facilitates CD36-TSP-$\alpha_{v\beta3}$–mediated uptake of apoptotic cells (25).

The $LXA_4$ receptor (ALXR) (also known as the FPRL-1 receptor) is a G-protein–coupled receptor (46) expressed in diverse cell types (4). $LXB_4$ does not compete with [$^3$H]$LXA_4$ for binding at the ALXR in PMN (47). In addition to binding $LXA_4$ 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 $\beta$ 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 M$\phi$ to either peptide stimulated phagocytosis to levels comparable to those seen with LX. Interestingly, additivity 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-$\beta_1$ 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 $LXA_4$ and $LXB_4$ 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-$LXB_4$ and the stable LX analogue 15(*R/S*)-methyl-$LXA_4$ (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-$\mu$g doses of 15(*R/S*)-methyl-$LXA_4$ and 15-epi-$LXB_4$ to that seen with 2-$\mu$g doses of the native $LXA_4$. 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 $\mu$g 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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