

# Lipoxin (LX)<sub>4</sub> and Aspirin-triggered 15-epi-LXA<sub>4</sub> Inhibit Tumor Necrosis Factor 1 $\alpha$ -initiated Neutrophil Responses and Trafficking: Regulators of a Cytokine-Chemokine Axis

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## Summary

The impact of lipoxin A<sub>4</sub> (LXA<sub>4</sub>) and aspirin-triggered lipoxins (ATLs) was investigated in tumor necrosis factor (TNF)- $\alpha$ -initiated neutrophil (polymorphonuclear leukocyte) responses in vitro and in vivo using metabolically stable LX analogues. At concentrations as low as 1–10 nM, the LXA<sub>4</sub> and ATL analogues each inhibited TNF- $\alpha$ -stimulated superoxide anion generation and IL-1 $\beta$  release by human polymorphonuclear leukocytes. These LXA<sub>4</sub>-ATL actions were time and concentration dependent and proved selective for TNF- $\alpha$ , as these responses were not altered with either GM-CSF- or zymosan-stimulated cells. TNF- $\alpha$ -induced IL-1 $\beta$  gene expression was also regulated by both anti-LXA<sub>4</sub> receptor antibodies and LXA<sub>4</sub>-ATL analogues. In murine air pouches, 15*R/S*-methyl-LXA<sub>4</sub> dramatically inhibited TNF- $\alpha$ -stimulated leukocyte trafficking, as well as the appearance of both macrophage inflammatory peptide 2 and IL-1 $\beta$ , while concomitantly stimulating IL-4 in pouch exudates. Together, these results indicate that both LXA<sub>4</sub> and ATL regulate TNF- $\alpha$ -directed neutrophil actions in vitro and in vivo and stimulate IL-4 in exudates, playing a pivotal role in immune responses.

Key words: eicosanoids • leukocytes • lipid mediators • antiinflammatory receptors • wound healing

Lipid and protein mediators of inflammation, such as cytokines and chemokines, have a profound impact on the formation and actions of each other (1). In particular, the cytokines TNF- $\alpha$  and IL-1 $\beta$  play major roles in inflammation, septic shock, and tissue injury. PMN perform a range of well appreciated, specialized functions, including chemotaxis, generation of reactive oxygen species (ROS)<sup>1</sup>, and biosynthesis of potent lipid mediators (2). In this regard, TNF- $\alpha$  stimulates PMN to transcribe and release cytokines such as IL-1 $\beta$ , enhances leukotriene biosynthesis, and upregulates adhesion molecules (3). As PMN represent ~70% of the peripheral blood leukocytes and are in many instances

the initial cell type recruited to interstitial sites, they are now considered a significant source of "proinflammatory" cytokines, including TNF- $\alpha$  and IL-1 $\beta$ . These as well as other PMN-derived cytokines and chemokines can, in turn, affect the course of inflammatory and immune responses (4). In certain clinical settings, including respiratory distress syndrome, myocardial reperfusion injury, gout, and rheumatoid arthritis (RA), PMN contribute to ongoing damage of host tissues (2, 5, 6). Thus, it is of interest to understand the complex relationships between lipid mediators and TNF- $\alpha$ -evoked PMN responses in order to gain insight for new approaches in controlling these events.

The contribution of leukotriene (LT)<sub>4</sub> in inflammation is well established in view of its potent ability to attract PMN. Another series of bioactive lipid mediators, termed lipoxins (LX) and aspirin-triggered lipoxins (ATLs), inhibits, within the nanomolar range, fMLP- and LTB<sub>4</sub>-stimulated PMN adhesion and transmigration (1, 7–9) and hence represent proposed counterregulatory signals operative in the resolution of inflammatory sites (10). In human tissues, three main pathways are known for LX generation. An in-

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<sup>1</sup>Abbreviations used in this paper: ATLs, aspirin-triggered lipoxins; ATL analogue, 15*R/S*-methyl-LXA<sub>4</sub>-methyl ester; LT, leukotriene; LX, lipoxin; LXA<sub>4</sub>, 5*S*,6*R*,15*S*-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; LXA<sub>4</sub> analogue, 16-phenoxylipoxin A<sub>4</sub> methyl ester; 15-epi-LXA<sub>4</sub>, 5*S*,6*R*,15*R*-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; MIP, macrophage inflammatory peptide; RA, rheumatoid arthritis; ROS, reactive oxygen species.

traluminal source of LX is exemplified by PMN-platelet interactions that utilize sequential transcellular biosynthetic routes with the PMN 5 lipoxygenase (LO) product LTA<sub>4</sub> and platelet 12-LO. The mucosal and/or interstitial source of these eicosanoids involves cell-cell interactions with leukocyte 5-LO and 15-LO present in, for example, eosinophils, gastrointestinal or tracheal epithelium controlled by IL-4 and IL-13 (for review see reference 1). The third and most recently elucidated pathway also represents a novel mechanism of action for aspirin that triggers the endogenous biosynthesis of 15*R* epimers of native LX, termed ATL, generated via transcellular biosynthesis (8).

LX are generated during cell-cell interactions via transcellular biosynthesis (1) and are produced *in vivo* during angioplasty and in immune complex glomerulonephritis (11). LXA<sub>4</sub> is also present in nasal lavage fluids of aspirin-sensitive asthmatics and is generated by leukocytes from patients with asthma and RA (12, 13). Like most autacoids and lipid mediators, LX are rapidly biosynthesized, act within a local microenvironment, and are rapidly enzymatically inactivated. To advance our understanding of LX and ATL roles *in vivo*, metabolically stable LX analogues were designed that resist rapid inactivation and mimic the *in vitro* actions of naturally occurring LX and ATL (14). Here, we report that these compounds are potent inhibitors of TNF- $\alpha$ -driven PMN-associated inflammatory events *in vitro* as well as *in vivo*. Moreover, LXA<sub>4</sub>-ATL inhibit macrophage inflammatory peptide (MIP)-2 and IL-1 $\beta$  yet stimulate the local appearance of IL-4 within exudates.

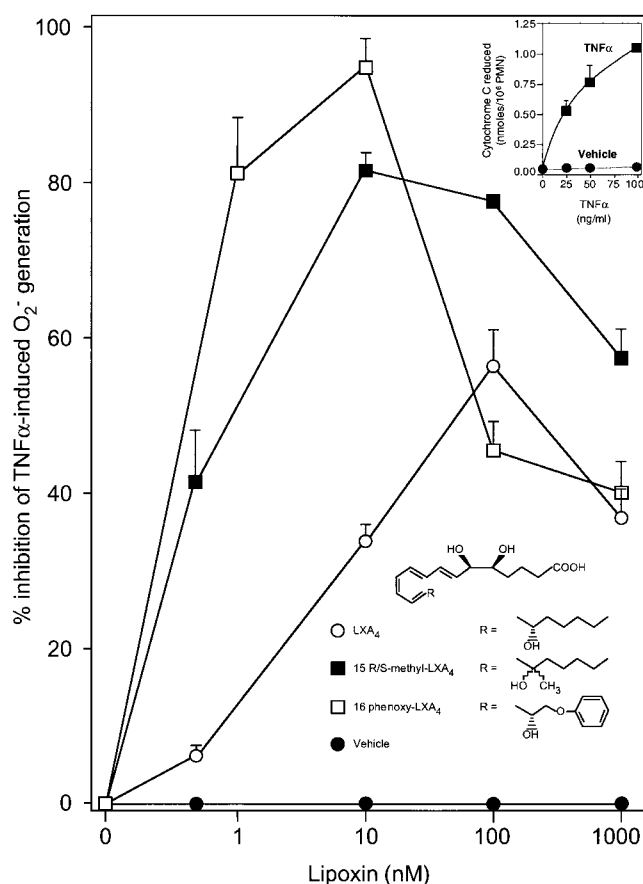
## Materials and Methods

Human and mouse rTNF- $\alpha$  and human rGM-CSF were obtained from Boehringer Mannheim. Dulbecco's PBS (Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free), RPMI 1640, and FCS were purchased from BioWhittaker, Inc. Ficoll-Hypaque was from Organon Teknika Corp., and HBSS was purchased from GIBCO BRL. BSA, dextran, antibiotics, l-glutamine, cytochrome C, superoxide dismutase, and zymosan were obtained from Sigma Chemical Co. The assessment of human IL-1 $\beta$  in supernatants was performed by using an immunometric assay with acetylcholine esterase (Cayman Chemical). Murine IL-1 $\beta$  was assessed using an ELISA from Endogen. ELISAs for IL-4 and IL-10 were from Amersham Corp.; MIP-2 and IL-13 ELISAs were from R & D Systems, Inc. LXA<sub>4</sub> and ATL metabolically stable analogues were prepared and characterized, including nuclear magnetic resonance spectroscopy, as in reference 14. Concentrations of each LX analogue were determined using an extinction coefficient of 50,000/M/cm just before each experiment and used as methyl esters. Where indicated, statistical analyses were performed using nonpaired *t* test (two-tailed), and significance was considered to be attained when *P* < 0.05.

**Preparation of Human PMN Suspensions and Superoxide Anion Generation.** Venous blood from healthy donors was collected under sterile conditions using acid citrate dextrose as an anticoagulant, and PMN were isolated as in reference 15. PMN were suspended in cold (4°C) Hank's medium (supplemented with 1.6 mM Ca<sup>2+</sup>, 0.1% FCS, 2 mM l-glutamine, 1% penicillin, and 2% streptomycin, pH 7.4). Cell preparations were >98% PMN, as determined by Giemsa-Wright staining. Cell viability was >98% for freshly isolated PMN and  $\geq$ 92% for PMN incubated for 20 h,

as determined by trypan blue exclusion using light microscopy. To examine superoxide production, PMN (10<sup>6</sup>/ml) were placed at 37°C (3 min) and then exposed to vehicle (0.1% ethanol) or synthetic LXA<sub>4</sub>, 15*R/S*-methyl LXA<sub>4</sub>, or 16-phenoxyl-LXA<sub>4</sub> for 5 min at 37°C. Before adding TNF- $\alpha$  (50 ng/ml), PMN were incubated with cytochrome C (0.7 mg/ml) for 10 min at 37°C. Superoxide dismutase-dependent reduction of cytochrome C was terminated by rapidly placing tubes in an ice water bath. The extent of cytochrome C reduction in each supernatant was determined at 550 nm in reference and compared with control values obtained when superoxide dismutase was added before a stimulus or vehicle control. Cytochrome C reduction was quantitated using the extinction coefficient of 21.1/mmol/liter.

**RNA Isolation and Northern Blot Analysis.** Total RNA extraction and Northern blot analyses were performed as in reference 7. pSM320 vector containing cDNA for IL-1 $\beta$  was purchased from American Type Culture Collection.



**Figure 1.** LXA<sub>4</sub> and ATL stable analogues inhibit TNF- $\alpha$ -stimulated superoxide generation by human neutrophils. Human PMN were incubated with vehicle alone or indicated concentrations of LXA<sub>4</sub>, 15*R/S*-methyl-LXA<sub>4</sub>, or 16-phenoxyl-LXA<sub>4</sub> for 5 min and then with TNF- $\alpha$  (50 ng/ml) for an additional 10 min. Values are the mean  $\pm$  SEM for LXA<sub>4</sub> (*n* = 3), 15*R/S*-methyl-LXA<sub>4</sub> (*n* = 4), or 16-phenoxyl-LXA<sub>4</sub> (*n* = 3). LXA<sub>4</sub> and analogues, at all concentrations tested, led to a statistically significant inhibition of TNF- $\alpha$ -induced IL-1 $\beta$  appearance (*P* < 0.01). Inset: TNF- $\alpha$  concentration-dependent superoxide production. Human PMN were incubated with indicated concentrations of TNF- $\alpha$ . Values are the mean  $\pm$  SEM (*n* = 3). TNF- $\alpha$  alone (50 ng/ml) gave 0.76  $\pm$  0.12 nmol cytochrome C-reduced cells per 10<sup>6</sup> cells compared with fMLP (10 nM), another physiologically relevant stimulus, that gave 6.02  $\pm$  0.05 nmol cytochrome C-reduced cells per 10<sup>6</sup> cells.

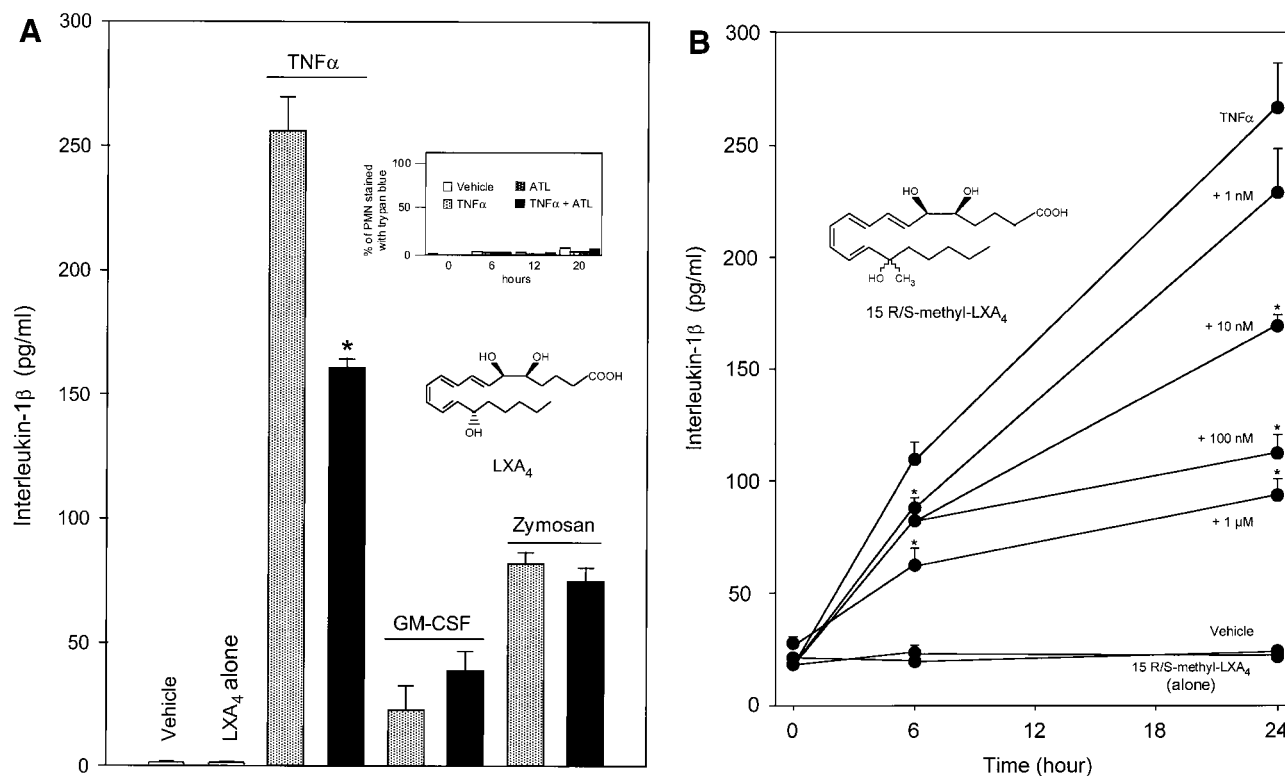
**Murine Air Pouches.** 6–8-wk-old male BALB/c mice were obtained from Taconic Farms, Inc. Air pouches were raised on the dorsum by subcutaneous injection of 3 ml of sterile air on days 0 and 3. All experiments were conducted on day 6 (16). Individual air pouches (one per mouse) were injected with vehicle alone (0.1% ethanol), TNF- $\alpha$ , 15*R/S*-methyl-LXA<sub>4</sub>, or TNF- $\alpha$  plus 15*R/S*-methyl-LXA<sub>4</sub>, and each was suspended in 1 ml endotoxin-free PBS immediately before injection into pouch cavities. At given intervals, the mice were killed, and individual air pouches were lavaged three times with sterile PBS (1 ml). The exudates were centrifuged at 2,000 rpm (5 min), and the supernatants were removed. Cell pellets were suspended in PBS (200  $\mu$ l) for enumeration and assessed for viability. 50  $\mu$ l of each cell suspension was mixed with 150  $\mu$ l 30% BSA and then centrifuged onto microscope slides at 500 rpm for 5 min using a cytospin centrifuge, air dried, and stained with Giemsa-Wright.

## Results and Discussion

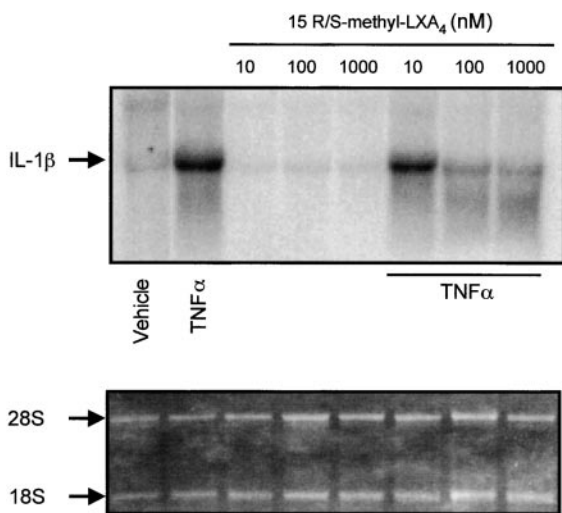
**Inhibition of TNF- $\alpha$ -stimulated Superoxide Generation.** TNF- $\alpha$ , although a modest agonist of O<sub>2</sub><sup>-</sup> generation by human PMN, is a physiologically relevant stimulus for the generation of ROS by nonadherent human PMN (17) that can play critical roles in local tissue injury during both inflammation and reperfusion (17–19). In Fig. 1, we evaluated the impact of LXA<sub>4</sub>- and ATL-related bioactive stable analogues on TNF- $\alpha$ -stimulated superoxide anion production. TNF- $\alpha$  gave a concentration-dependent increase in

superoxide anion dependence (Fig. 1, inset) with nonadherent PMN; therefore, TNF- $\alpha$  (50 ng/ml) was used to examine the analogues. Native LXA<sub>4</sub> and the analogues (15*R/S*-methyl-LXA<sub>4</sub> and 16 phenoxy-LXA<sub>4</sub>) inhibited TNF- $\alpha$ -stimulated superoxide anion generation in a concentration-dependent fashion. Their rank order of potency at 10 nM was 15*R/S*-methyl-LXA<sub>4</sub> (81.3  $\pm$  14.1% inhibition)  $\approx$  16-phenoxy-LXA<sub>4</sub> (93.7  $\pm$  3.2%) > LXA<sub>4</sub> (34.3  $\pm$  2.3%). 15*R/S*-methyl-LXA<sub>4</sub> covers both LXA<sub>4</sub> and ATL in structure, and 16-phenoxy-LXA<sub>4</sub> is an LXA<sub>4</sub> analogue (Fig. 1). Each analogue competes at the LXA<sub>4</sub>R (7). LXA<sub>4</sub>, 15*R/S*-methyl-LXA<sub>4</sub>, and 16 phenoxy-LXA<sub>4</sub>, at concentrations up to 1  $\mu$ M added to cells alone, did not stimulate generation of ROS (data not shown). 15*R/S*-methyl-LXA<sub>4</sub> and 16-phenoxy-LXA<sub>4</sub> were approximately three times more potent than native LXA<sub>4</sub> and proved to be powerful inhibitors of TNF- $\alpha$ -stimulated superoxide generation by PMN. However, neither LXA<sub>4</sub> nor its analogues inhibit PMA (100 nM)- or fMLP (100 nM)-stimulated O<sub>2</sub><sup>-</sup> production ( $n = 3$ ; data not shown). Inhibition of ROS by LXA<sub>4</sub> and its analogues is of interest in a context of ischemia/reperfusion, where ROS are held to be primary mediators of tissue injury (15).

**Suppression of TNF- $\alpha$ -stimulated IL-1 $\beta$  Release.** PMN express and release interleukin-1 $\beta$ , which is a potent pro-inflammatory cytokine (20). Therefore, we next investigated the actions of native LXA<sub>4</sub> and its analogues on TNF-



**Figure 2.** LXA<sub>4</sub> and stable analogues inhibit TNF- $\alpha$ -induced IL-1 $\beta$  production in human neutrophils. (A) PMN were incubated with TNF- $\alpha$  [10 ng/ml]; stippled bar) plus vehicle or TNF- $\alpha$  plus LXA<sub>4</sub> (100 nM; black bar) as denoted for 20 h at 37°C and 5% CO<sub>2</sub>. Supernatants were collected, and IL-1 $\beta$  was quantitated by ELISA. Results are expressed as mean  $\pm$  SD of duplicates and are from one experiment representative of  $n = 3$ . (B) PMN were incubated for indicated periods of time in the presence of increasing concentrations of 15*R/S*-methyl-LXA<sub>4</sub>. Values represent the mean  $\pm$  SEM,  $n = 3$ . At all time intervals tested, TNF- $\alpha$  induced a significant appearance of IL-1 $\beta$  over vehicle-treated cells (\* $P < 0.01$ ).



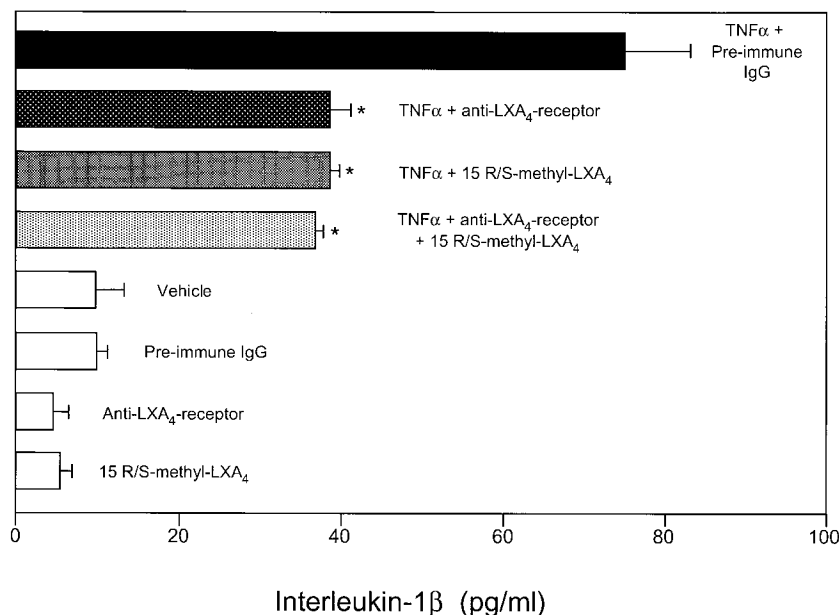
**Figure 3.** 15*R/S*-methyl-LXA<sub>4</sub> downregulates TNF- $\alpha$ -triggered IL-1 $\beta$  gene expression. PMN were incubated with either 0.1% ethanol (vehicle) or 15*R/S*-methyl-LXA<sub>4</sub> at 10, 100, and 1,000 nM, in the presence or absence of TNF- $\alpha$  (10 ng/ml), for 6 h at 37°C. Northern blot analyses were performed in order to detect IL-1 $\beta$  mRNA. The results presented are from one experiment, which is representative of two others performed with different donors.

$\alpha$ -induced IL-1 $\beta$  release. Incubation of PMN with physiologically relevant concentrations of TNF- $\alpha$ , GM-CSF, or phagocytic particles (zymosan) resulted in a concentration-dependent increase in the levels of IL-1 $\beta$  present in supernatants. Approximate EC<sub>50</sub> for each agonist were: TNF- $\alpha$ , 10 ng/ml; GM-CSF, 10 U/ml; and zymosan, 100  $\mu$ g/ml. Native LXA<sub>4</sub> specifically inhibited TNF- $\alpha$ -induced IL-1 $\beta$  release (Fig. 2 A), whereas similar amounts of IL-1 $\beta$  were released in the presence or absence of LXA<sub>4</sub> when PMN were exposed to either GM-CSF or zymosan. The viability of PMN exposed to ATL or TNF- $\alpha$  was examined using trypan blue exclusion. PMN exposed to these agents did not

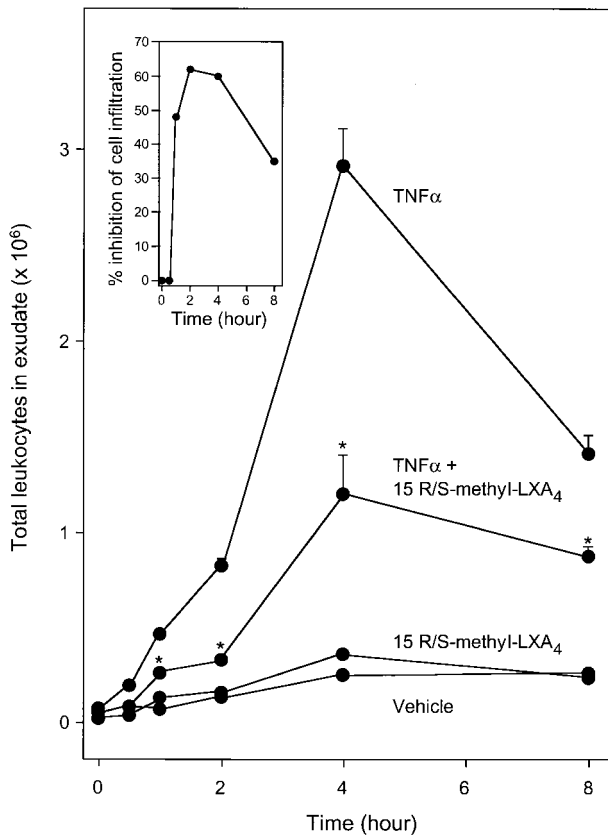
dramatically increase their staining (Fig. 2 A, inset), suggesting that the ATL did not reduce PMN viability during the time courses of these experiments.

PMN were exposed to increasing concentrations of 15*R/S*-methyl-LXA<sub>4</sub>, 16-phenoxy-LXA<sub>4</sub>, or native LXA<sub>4</sub> in the presence of TNF- $\alpha$  (10 ng/ml) or vehicle alone. At a concentration of 100 nM, 15*R/S*-methyl-LXA<sub>4</sub> inhibited ~60% of IL-1 $\beta$  release, and 16-phenoxy-LXA<sub>4</sub> at equimolar levels gave ~40% inhibition (values comparable to those obtained with native LXA<sub>4</sub>; data not shown). Time course and concentration dependence were carried out with 15*R/S*-methyl LXA<sub>4</sub> (Fig. 2 B). At 10 nM, 15*R/S*-methyl-LXA<sub>4</sub> gave clear, statistically significant inhibition, which was evident within 6 h and more prominent after 24 h (Fig. 2 B). Inhibition of IL-1 $\beta$  by these LX analogues was, at least in part, the result of a downregulation in gene expression, because the IL-1 $\beta$  messenger RNA levels in cells treated with TNF- $\alpha$  (10 ng/ml) plus 15*R/S*-methyl-LXA<sub>4</sub> (100 nM) were decreased by ~60% when compared with cells treated with TNF- $\alpha$  alone (Fig. 3). Therefore, as IL-1 $\beta$  and TNF- $\alpha$  are two cytokines that are considered important in inflammation, the inhibition of IL-1 $\beta$  observed (Figs. 1 and 2) suggested that 15*R/S*-methyl-LXA<sub>4</sub> might exert a potent *in vivo* anticytokine action (*vide infra*).

**Involvement of LXA<sub>4</sub>R.** To investigate whether LXA<sub>4</sub>R was involved in the regulation of TNF- $\alpha$ -stimulated IL-1 $\beta$  release, the rabbit polyclonal antibodies against a portion of the third extracellular domain (ASWGGTPEERLK) of LXA<sub>4</sub>R prepared earlier (21) were used. PMN were incubated with ~50  $\mu$ g/ml of either preimmune protein A-purified IgG or IgG directed against LXA<sub>4</sub>R for 1 h at 4°C before exposure to TNF- $\alpha$  (10 ng/ml) and 15*R/S*-methyl-LXA<sub>4</sub> (100 nM). Anti-LXA<sub>4</sub>R antibodies prevented IL-1 $\beta$  release by TNF- $\alpha$ , suggesting that the third extracellular loop plays a crucial role in LXA<sub>4</sub>R activation (Fig. 4). 15*R/S*-methyl-LXA<sub>4</sub> inhibited ~50% of IL-1 $\beta$



**Figure 4.** Involvement of LXA<sub>4</sub>R. PMN were incubated with either IgG purified from preimmune serum (50  $\mu$ g/ml) or anti-LXA<sub>4</sub>R (50  $\mu$ g/ml) for 1 h at 4°C and then exposed to agonists for 12 h at 37°C and 5% CO<sub>2</sub>. Values are expressed as mean  $\pm$  SD from an experiment performed in triplicate, which is representative of three distinct experiments, each performed with different donors (\**P* < 0.01).



**Figure 5.** Inhibition of TNF- $\alpha$ -induced PMN infiltration in murine air pouches. 1 ml of sterile PBS containing 0.1% ethanol, TNF- $\alpha$ , 15R/S-methyl-LXA<sub>4</sub>, or TNF- $\alpha$  plus 15R/S-methyl-LXA<sub>4</sub> was injected into the pouches, and the exudates were collected at indicated time periods. The total number of leukocytes was counted as described in Materials and Methods. The results are expressed as mean  $\pm$  SEM from three different mice for each point. At all time intervals, TNF- $\alpha$  induced a significant leukocyte infiltration into the air pouch cavity ( $P < 0.05$ ). \*Statistically different from TNF- $\alpha$ -treated, vehicle-, or 15R/S-methyl-LXA<sub>4</sub>-treated cells ( $P < 0.01$ ).

release. When added together, anti-LXA<sub>4</sub>R antibodies and 15R/S-methyl-LXA<sub>4</sub> in the presence of TNF- $\alpha$  did not further inhibit IL-1 $\beta$  appearance, and neither anti-LXA<sub>4</sub>R antibodies nor 15R/S-LXA<sub>4</sub> alone stimulated significant amounts of IL-1 $\beta$  to appear in supernatants. The results of these experiments are twofold: first, they indicated that the inhibitory action of 15R/S-methyl-LXA<sub>4</sub> is transduced via LXA<sub>4</sub>R and second, that the anti-LXA<sub>4</sub>R antibodies alone activate LXA<sub>4</sub>R and lead to inhibition of IL-1 $\beta$  release.

**Inhibition of TNF- $\alpha$ -directed Leukocyte Trafficking In Vivo.** As TNF- $\alpha$  evokes leukocyte infiltration in a chemokine-dependent fashion in the murine six-day air pouch (16, 22), we evaluated the impact of 15R/S-methyl-LXA<sub>4</sub> in this model to determine whether LXA<sub>4</sub> or ATL also intersects the cytokine-chemokine axis in vivo. 15R/S-methyl-LXA<sub>4</sub> is the most subtle modification to native LXA<sub>4</sub> and ATL structure, with addition of a methyl at carbon 15. Murine TNF- $\alpha$  (10 ng/ml) caused a transient infiltration of leukocytes to the air pouch in a time-dependent fashion, with maximal accumulation at 4 h. 15R/S-methyl-LXA<sub>4</sub> at 25 nmol

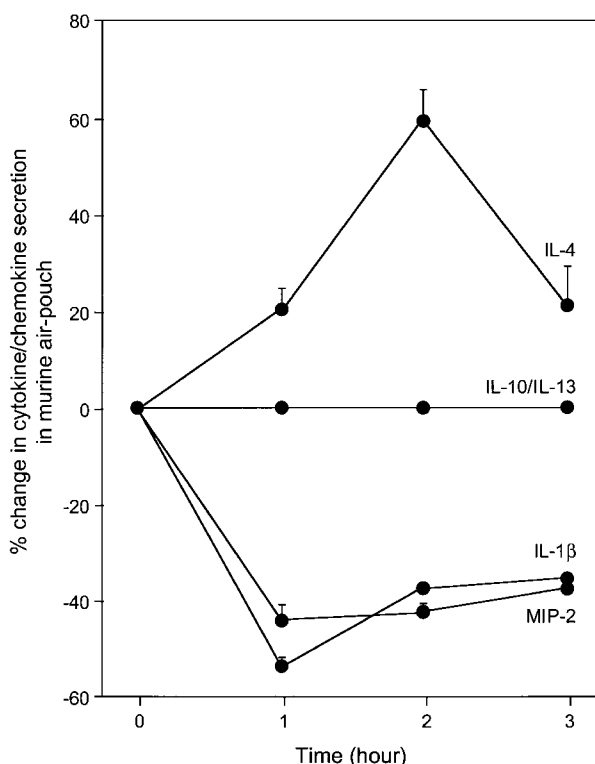
**Table I.** TNF- $\alpha$ -induced Leukocyte Infiltration in Murine Air Pouches: Inhibitory Action of 15R/S-methyl-LXA<sub>4</sub>

Injection	Number of leukocytes present in pouch ( $\times 10^6$ )		
	Neutrophils	Eosinophils/ basophils	Monocytes/ macrophages
TNF- $\alpha$	2.40 $\pm$ 0.10*	0.30 $\pm$ 0.01*	0.20 $\pm$ 0.01*
15R/S-methyl-LXA <sub>4</sub> + TNF- $\alpha$	0.98 $\pm$ 0.10 <sup>‡</sup> (59.1%)	0.13 $\pm$ 0.01 <sup>‡</sup> (56.0%)	0.10 $\pm$ 0.01 <sup>‡</sup> (50%)
15R/S-methyl-LXA <sub>4</sub>	0.25 $\pm$ 0.01	0.03 $\pm$ 0.01	0.14 $\pm$ 0.01*
Vehicle	0.30 $\pm$ 0.01	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01

Air pouches were raised as described in Materials and Methods. Each mouse was injected with 1 ml PBS containing vehicle (0.1% ethanol), TNF- $\alpha$  (10 ng), 15R/S-methyl-LXA<sub>4</sub> (25 nmol), or TNF- $\alpha$  plus 15R/S-methyl-LXA<sub>4</sub>. Leukocyte infiltration was determined 4 h after injection. Results present the mean  $\pm$  SEM of three different mice. Percent inhibition is indicated in parentheses. Statistically different from \*vehicle-injected mice ( $P < 0.01$ ) and <sup>‡</sup>TNF- $\alpha$ -injected mice ( $P < 0.01$ ).

inhibited the TNF- $\alpha$ -stimulated recruitment of leukocytes to the air pouch by 62% (Fig. 5). Inhibition was evident at 1 h and maximal between 2 and 4 h. At these intervals, a  $>60\%$  reduction in leukocyte infiltration was noted that remained significantly reduced at 8 h (Fig. 5, inset). Injection of pouches either with vehicle or the analogue alone did not cause a significant leukocyte infiltration. Also, inflammatory exudates were collected 4 h after injection with vehicle alone, TNF- $\alpha$ , 15R/S-methyl-LXA<sub>4</sub> alone, or TNF- $\alpha$  plus 15R/S-methyl-LXA<sub>4</sub>, and cell types were enumerated. In the six-day pouches given TNF- $\alpha$ , PMN constituted the major cell type present within the exudates at 4 h and ranged from 80 to 85% of total cell number. Administration of both 15R/S-methyl-LXA<sub>4</sub> and TNF- $\alpha$  into the six-day air pouch cavity inhibited migration of PMN and eosinophils/basophils as well as mononuclear cells (Table I). Of interest is the finding that administration of 15R/S-methyl-LXA<sub>4</sub> alone evoked a small but statistically significant increase in mononuclear cell influx (Table I), a result that is consistent with earlier in vitro observations (23) in which specific stimulation of monocyte and inhibition of PMN chemotaxis have been observed.

**Cytokine-Chemokine Profiles.** Because MIP-2 is the major chemokine involved in recruiting PMN to the air pouch after injection of TNF- $\alpha$  (16), we determined the action of 15R/S-methyl-LXA<sub>4</sub> in this TNF- $\alpha$ -induced chemokine-cytokine axis. MIP-2 and IL-1 $\beta$  are important proinflammatory cytokines, and IL-4, IL-10, and IL-13 possess immunomodulatory properties (24, 25). Exudates from selected time intervals were collected, and cell-free supernatants were assessed for the presence of these murine cytokines. TNF- $\alpha$  induced maximal detectable amounts of MIP-2 and IL-1 $\beta$  within 90 min (data not shown). 15R/S-methyl-LXA<sub>4</sub> (25 nmol) inhibited TNF- $\alpha$ -stimulated MIP-2 and IL-1 $\beta$  release by 48 and 30%, respectively (Fig. 6). 15R/S-



**Figure 6.** *15R/S*-methyl-LXA<sub>4</sub> redirects the TNF- $\alpha$ -induced cytokine-chemokine profile in vivo. Experiments were conducted as described in the Fig. 5 legend. Quantitation for IL-1 $\beta$ , IL-4, IL-10, IL-13, and MIP-2 was performed using ELISA with air pouch cell-free exudates. The results are expressed as mean  $\pm$  SEM from three different mice for each time point. Changes in IL-1 $\beta$ , MIP-2, and IL-4 were significant at all tested time intervals ( $P < 0.01$ ). At 1 h, air pouches injected with TNF- $\alpha$  alone generated  $384 \pm 12$  pg/pouch of MIP-2 and  $14.9 \pm 2.3$  pg/pouch of IL-1 $\beta$ . *15R/S*-methyl-LXA<sub>4</sub> alone induced  $42.7 \pm 0.7$  pg/pouch of IL-4.

methyl-LXA<sub>4</sub> alone in the air pouch did not stimulate MIP-2 or IL-1 $\beta$  release. In sharp contrast, *15R/S*-methyl-LXA<sub>4</sub> stimulated the appearance of IL-4 within the exudates. This stimulation of IL-4 was observed both in the absence as well as the presence of TNF- $\alpha$ . Neither IL-10 nor IL-13 was detected within the pouch exudates. These results demonstrate that administration of *15R/S*-methyl-LXA<sub>4</sub> modified the cytokine-chemokine axis in TNF- $\alpha$ -initiated acute inflammation, and, interestingly, this reorientation of the cyto-

kine-chemokine axis paralleled the reduction in leukocyte infiltration.

Several different strategies have been explored in an attempt to attenuate undesirable action of TNF- $\alpha$  in inflammatory diseases and ischemia/reperfusion injury, including treatment of patients suffering from RA with rTNF- $\alpha$ R linked to human Ig as a fusion protein (26). Different steroidal and nonsteroidal drugs (27) to alleviate the pain and the severity of inflammatory responses are extensively used. However, certain clinical settings, such as reperfusion injury, are still not well controlled, and new therapeutic agents are needed. Our results indicate that LXA<sub>4</sub> and ATL, as evidenced by the actions of their metabolically stable analogues (16-phenoxy-LXA<sub>4</sub> and *15R/S*-methyl-LXA<sub>4</sub>), are potent cytokine-regulating lipid mediators that can also impact the course of inflammation initiated by TNF- $\alpha$  and IL-1 $\beta$ . These two cytokines are considered to be key components in orchestrating the rapid inflammatory-like events in ischemia/reperfusion (within minutes to hours) and are major cytokines in RA and many other chronic diseases. Interestingly, in an exudate and skin wound model, *15R/S*-methyl-LXA<sub>4</sub> not only inhibited the TNF- $\alpha$ -elicited appearance of IL-1 $\beta$  and MIP-2 but also concomitantly stimulated IL-4 (Figs. 5 and 6). This represents the first observation that lipoxins induce upregulation of a potential "antiinflammatory" cytokine such as IL-4. Hence, it is of particular interest that IL-4 inhibits PMN influx in acute antibody-mediated inflammation (28) and inhibits H<sub>2</sub>O<sub>2</sub> production by IFN- $\gamma$ -treated human monocytes (29). IL-4 is also an active antitumor agent and, most recently, was shown to be a potent inhibitor of angiogenesis (25). It is thus likely that the increase in IL-4 levels stimulated by metabolically stable LX analogues may in part mediate some of the in vivo impact of LXA<sub>4</sub> and aspirin-triggered 15-epi-LXA<sub>4</sub>, a finding that provides a new understanding of the relationship between antiinflammatory cytokines and lipid mediators.

In conclusion, LXA<sub>4</sub> and ATL appear to be involved in controlling both acute as well as chronic inflammatory responses. The results presented here support the notion that aspirin may exert its beneficial action in part via the biosynthesis of endogenous ATL that can in turn act directly on PMN and/or the appearance of IL-4. Thus, LX-ATL can protect host tissues via multilevel regulation of proinflammatory signals.

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