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Aspirin-Triggered Lipoxin A_4 and B_4 Analogs Block Extracellular Signal-Regulated Kinase-Dependent TNF-α Secretion from Human T Cells[1]

Amiram Ariel,* Nan Chiang,* Makoto Arita,* Nicos A. Petasis,† and Charles N. Serhan[2]*

Lipoxins (LX) and their aspirin-triggered 15-epimer endogenous isoforms are endogenous anti-inflammatory and pro-resolution eicosanoids. In this study, we examined the impact of LX and aspirin-triggered LXA_4-stable analogs (ATLa) on human T cell functions. 15-epi-16-(*p*-fluoro)phenoxy-LXA_4 ($ATLa_1$) blocked the secretion of TNF-α from human PBMC after stimulation by anti-CD3 Abs, with the IC_{50} value of $\approx$0.05 nM. A similar action was also exerted by the native aspirin-triggered 15-epi-LXA_4, a new 15-epi-16-(*p*-trifluoro)phenoxy-LXA_4 analog ($ATLa_2$), as well as LXB_4, and its analog 5-(*R/S*)-methyl-LXB_4. The LXA_4 receptor (ALX) is expressed in peripheral blood T cells and mediates the inhibition of TNF-α secretion from activated T cells by $ATLa_1$. This action was accomplished by inhibition of the anti-CD3-induced activation of extracellular signal-regulated kinase, which is essential for TNF-α secretion from anti-CD3-activated T cells. These results demonstrate novel roles for LX and aspirin-triggered LX in the regulation of T cell-mediated responses relevant in inflammation and its resolution. Moreover, they provide potential counterregulatory signals in communication(s) between the innate and acquired immune systems. ***The Journal of Immunology,*** **2003, 170: 6266–6272.**

Specific lipid-derived mediators can exert opposing actions on T cell proinflammatory functions (1–3). The key role of the classic eicosanoids, such as the prostanoids in the regulation of inflammatory and autoimmune disorders, is also well appreciated (4). In rheumatoid arthritis, which is mediated in part by the activation and recruitment of Ag-specific lymphocytes (5), leukotriene (LT)[3] B_4 generated locally can control T cell activation, regulating the inflammatory response (6, 7).

In humans, lipoxin (LX) A_4 and B_4 are generated mainly via transcellular biosynthesis during cell-cell interactions and possess anti-inflammatory and pro-resolving properties both in vitro and in vivo (for a recent review, see Ref. 8). LXs are formed in various inflammatory conditions. For example, LXA_4 is produced by polymorphonuclear leukocytes (PMN) from asthmatic patients (9) as well as during the resolution of acute inflammation (10) and in murine spleens after microbial challenge (11). Synthetic metabolically stable LX analogs with prolonged bio-half-life were designed which mimic LX actions and exhibit potent anti-inflammatory actions. Some of these actions include inhibition of neutrophil (PMN) and eosinophil chemotaxis, inhibition of PMN transmigration across epithelial and endothelial cell layers, and of O_2^- and IL-1β production in neutrophils, as well as inhibition of TNF-α-induced IL-8 secretion from colon epithelial cells (reviewed in Ref. 8). LX-stable analogs also reduce LTB_4-induced infiltration of PMN into mouse ears (12) and TNF-α-induced infiltration of PMN into murine dorsal air pouches (13). In addition, LXA_4 inhibits T cell expression of the LTB_4 receptor (BLT) in guinea pigs (14) and events relevant in T cell-mediated inflammatory processes and autoimmune disorders, such as colitis, and chronic asthma (15, 16).

Aspirin inhibits prostaglandin generation and can also impact LX-sensitive responses. This involves two cyclooxygenase isoforms, namely, cyclooxygenase 1 and 2. The physiological function of cyclooxygenase 2 is still not fully understood; however, its acetylation by aspirin modifies its activity to generate 15*R*-hydroxyeicosatetraenoic acid, which can be oxygenated to produce 15-epi-LXA_4, termed aspirin-triggered LX (ATL; reviewed in Ref. 8). Also of interest, aspirin shares anti-inflammatory actions with 15-epi-LXA_4, such as inhibition of IL-12 production from dendritic cells (11, 17), but the 15-epi-LXA_4 analog acts at much lower concentrations (100–1000×) than aspirin when blocking PMN infiltration to the ears of mice during inflammation (13).

TNF-α is a very potent cytokine produced by various cells and is critical for the development of a complete inflammatory response in various autoimmune disorders (18). T cells secrete TNF-α upon stimulation by CD3 cross-linking that mimics the engagement of the TCR by a MHC II-peptide complex. TNF-α regulates various lymphocyte functions, such as cell proliferation and IL-2R expression (19), and is critical for the progression of rheumatoid arthritis (20). The substantial role played by TNF-α during inflammation led to the development of different strategies to block its activity (21), although not all patients respond to these treatments, and they could be accompanied with severe side effects, such as the induction of tuberculosis (22). In this report, we

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[3] Abbreviations used in this paper: LT, leukotriene; LX, lipoxin; ALX, LXA_4 receptor; ATL, aspirin-triggered 15-epi-lipoxin; $ATLa_1$, ATL A_4 stable analog 15-epi-16-(*p*-fluoro)phenoxy-LXA_4; $ATLa_2$, 15-epi-16-(*p*-trifluoro)phenoxy-LXA_4; BLT, leukotriene B_4 receptor; ERK, extracellular signal-regulated kinase; LXA_4, 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXB_4, 5*S*,14*R*,15*S*-trihydroxy-6,8,12-*trans*-10-*cis*-eicosatetraenoic acid; MAPK, mitogen-activated protein kinase; PBTC, peripheral blood T cell; PMN, polymorphonuclear leukocytes; MD, mean density.

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examined the ability of LX and ATL to modulate extracellular signal-regulated kinase (ERK)-dependent TNF-α secretion from T cells. Both 15-epi-LXA_4 and LXB_4 and their stable analogs blocked the secretion of TNF-α by T cells stimulated via their TCR complex. We also found that the ALX is expressed in peripheral blood T cells (PBTC), and that, in activated T cells that express ALX, TNF-α secretion is inhibited specifically by $ATLa_1$. Moreover, we report that anti-CD3-induced ERK activation is crucial for TNF-α secretion and is regulated by $ATLa_1$.

Materials and Methods

Reagents

The following materials were obtained as indicated: BSA, Histopaque-1077, and alkaline phosphatase substrate from Sigma-Aldrich (St. Louis, MO); RPMI 1640, FBS, glutamine, penicillin, and streptomycin from Bio-Whittaker (Walkersville, MD); G418 sulfate from Cellgro (Herndon, VA); purified and biotinylated mouse anti-human TNF-α from BD PharMingen (San Diego, CA); and streptavidin-conjugated alkaline phosphatase from Jackson ImmunoResearch (West Grove, PA). The synthetic 15-epi-LXA_4, LXB_4, and their stable analogs used in the presented experiments were prepared by total organic synthesis and characterized, including magnetic resonance spectroscopy, as in the studies of Serhan et al. (23) and Maddox et al. (24), respectively.

Human T cell incubations

PBMC were separated from venous blood of healthy volunteers as previously described (25). Briefly, The blood was loaded over Histopaque-1077 (15 ml) and centrifuged at 2000 rpm for 30 min without a break. Then the interface was collected and washed twice with PBS. Next, platelets were depleted by centrifugation at 700 rpm for 15 min. The acquired PBMC were used for in vitro experiments or further separated to yield a purified T cell preparation. To this end, PBMC were incubated in culture medium (RPMI 1640, 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) on a plastic surface for 2 h for monocyte depletion. Then the cells were collected and resuspended in column buffer ($50–100 \times 10^6$ cells in 0.5 ml) and separated by a human $CD3^+$ enrichment column (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions, and cultured. T cell purity was typically >92%.

PBMC were treated with the indicated concentrations of the following LX analogs: 15-epi-LXA_4, 15-epi-16-(*p*-fluoro)phenoxy-LXA_4 ($ATLa_1$), 15-epi-16-(*p*-trifluoro)phenoxy-LXA_4 ($ATLa_2$), LXB_4, or 5-(*R*/*S*)-methyl-LXB_4. Alternatively, the cells were incubated with the mitogen-activated protein/ERK kinase (MEK) inhibitor U0126 (Cell Signaling Technology, Beverly, MA) or the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (Biomol, Plymouth Meeting, PA) for 6 h at 37°C and then seeded over anti-CD3 (2 μg/ml)-coated wells and cultured for 65 h. Then the supernatants were collected from the wells and evaluated for their TNF-α content by standard ELISA.

$CD4^+$ Jurkat T cells were transfected with pcDNA3 (Invitrogen, Carlsbad, CA) containing a neomycin resistance gene and full-length ALX cDNA or pcDNA3 alone (mock) using the SuperFect reagent (Qiagen, Chatsworth, CA) (26) and cultured in culture medium supplemented with G418 (0.5 mg/ml) to select for cells that express ALX. After the cell culture recovered from the selection, the expression of ALX mRNA in the corresponding cells was confirmed by RT-PCR. The culture was maintained in selective conditions and cells were harvested for experiments. Jurkat cells transfected with ALX (J/ALX) or mock (J/mock) constructs were incubated for 6 h with vehicle, $ATLa_1$, or LXB_4 at 0.001–0.1 nM and then seeded over anti-CD3 (2 μg/ml)-coated wells and activated by PMA (20 nM). TNF-α secretion to the medium was determined after 16 h.

Proliferation assay

Human T cells were loaded with 10 μM CFSE for 30 min, washed, and then treated by $ATLa_1$ (10 nM) or vehicle for 6 h. Then the cells (0.5×10^6 cells/well) were incubated over anti-CD3- or BSA-coated 96-well plates for 65 h at 37°C, with 5% CO_2, in a humidified atmosphere. Then the cells were collected and analyzed by FACSort (BD Biosciences, Franklin Lakes, NJ).

RT-PCR

Total RNA was extracted from human PBTC or PMN with TRIzol reagent (Life Technologies, Grand Island, NY). RNA levels were determined and equal amounts from each sample were taken for RT-PCR using the Titan One Tube system (Roche, Mannheim, Germany) with the following ALX-specific primers: sense, 5′-CACCAGGTGCTGCTGGCAAG-3′ (corresponding to the immediate 5′ side of the start codon) and antisense, 5′-AATATCCCTGACCCCATCCTCA-3′ (corresponding to the immediate 3′ side of the stop codon) as described previously (27). After 40 cycles of PCR, amplified cDNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and photographed. DNA sequencing was conducted by the Brigham and Women's Hospital sequencing facility.

Western blotting

Freshly isolated T cells or J/ALX cells were incubated with $ATLa_1$ (10 nM) or vehicle for 6 h at 37°C. Next, the cells (5×10^6/0.5 ml) were seeded over anti-CD3 (2 μg/ml)-coated wells for different times. Then the cells were moved to ice and added with cold PBS to stop signaling events. The cells were transferred to tubes and lysed by lysis buffer (10 mM Tris (pH 7.5), 30 mM KCl, 3 mM NaCl, 2 mM EDTA, 1 tablet/ml protease inhibitor mixture (Roche), 1% Triton X-100, and phosphatase inhibitor mixture II (1:1000; Sigma-Aldrich)) for 20 min at 4°C. The cytoplasmic proteins were collected and added with sample buffer and equal amounts of protein from each sample were run by SDS-PAGE on a 10% gel. Then the proteins were transferred to a nitrocellulose membrane and blocked by 1% BSA for 1 h. Next, the membrane was blotted with anti-phospho-ERK or -total-ERK (28) diluted 1/1000 in TBST and exposed to x-ray film, which was developed and scanned. The appropriate bands were analyzed using the NIH image program to determine the ERK activation index. Blotting with anti-total-ERK was done with the same membrane after it was stripped from the anti-phospho-ERK Ab by incubation for 20 min with 0.1% Ponceau S in 5% acetic acid solution and washing with TBST. Activation index was calculated in the following way: ((mean density (MD) phospho-ERK time X − MD phospho-ERK background)/(MD total-ERK time X − MD total-ERK background))/((MD phospho-ERK time 0 − MD phospho-ERK background)/(MD total ERK time 0 − MD total ERK background)).

Statistical analyses

Results were analyzed using unpaired one-tailed distribution Student *t* tests, and significance was assigned at $p < 0.05$.

Results

$ATLa_1$ modulates TNF-α secretion from PBMC stimulated by anti-CD3 Abs

Immobilized anti-CD3 Abs stimulate T cells to secrete TNF-α (29). To evaluate the actions of LX analogs on maximal T cell response to anti-CD3, a time course for TNF-α secretion from PBMC after anti-CD3 stimulation was initially performed. TNF-α in the culture medium reached maximal levels (90 ± 19 ng/ml) 3 days after exposure to anti-CD3 and then declined at day 4 (Fig. 1*A*). To determine the effective concentration of $ATLa_1$ required to modulate TNF-α secretion, PBMC were exposed to increasing concentrations (0.01–10 nM) of $ATLa_1$ and then activated by anti-CD3. $ATLa_1$ significantly ($p < 0.005$) inhibited the secretion of TNF-α from anti-CD3-activated PBMC in a concentration-dependent manner with maximal inhibition (86.8 ± 3.2%) at 1 nM (Fig. 1, *B* and *C*). Of interest, isolated PBTC also gave reduced levels of TNF-α when exposed to the same conditions in parallel (182.7 ± 32.2 ng/ml of TNF-α secreted with anti-CD3 stimulation alone, 79.05 ± 6.1% inhibition with 0.1 nM $ATLa_1$; $n = 3$). The reduction in TNF-α secretion could be a nonspecific byproduct of inhibition of T cell proliferation in response to CD3 cross-linking. To exclude that possibility, we determined whether $ATLa_1$ inhibits cellular division. Our results (Fig. 1*D*) indicate that $ATLa_1$ did not inhibit T cell proliferation. In addition, we found that the secretion of IFN-γ from anti-CD3-stimulated PBMC was not inhibited by $ATLa_1$ (data not shown). Thus, TNF-α secretion from PBMC and PBTC stimulated through their TCR is potently and specifically blocked by $ATLa_1$.

Both ATL and LXB_4 analogs inhibit TNF-α secretion

LXA_4 and B_4 are subjected to rapid enzymatic inactivation. Hence, various metabolically stable analogs of 15-epi-LXA_4 and LXB_4

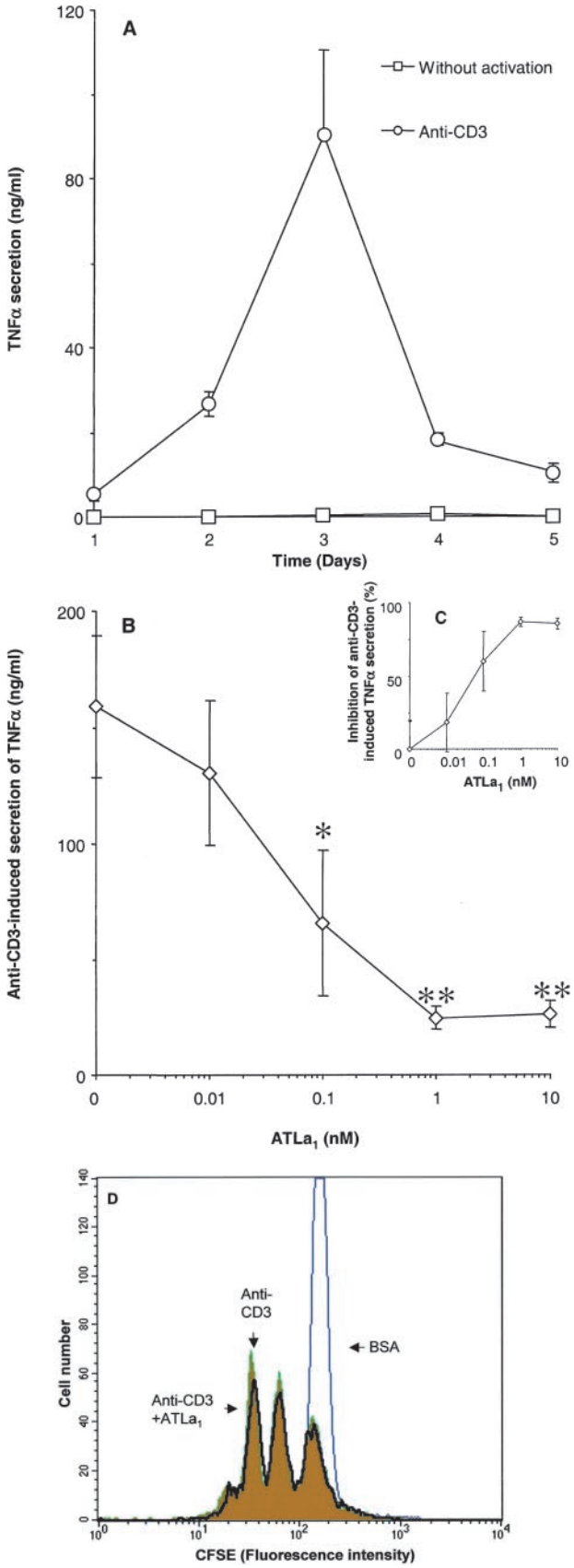

FIGURE 1. Modulation by ATL analog of TNF-α secretion from PBMC after stimulation by anti-CD3 Abs. *A*, Human PBMC were incubated over immobilized anti-CD3 Abs (2 μg/ml) for the indicated periods and then the supernatants were collected from the wells and their TNF-α content was examined using standard ELISA. *B*, Human PBMC were incubated with the indicated concentrations of $ATLa_1$ or vehicle (0.05% ethanol) for 6 h and then stimulated by immobilized anti-CD3 for 65 h at 37°C with 5% CO_2 in a humidified atmosphere. Then the supernatants were collected and TNF-α levels were determined by standard ELISA. Inhibition percentage from vehicle plus anti-CD3-treated cells was calculated and the results are presented in *C*. *, $p < 0.05$; **, $p < 0.005$ vs vehicle treatment. Results are mean ± SE representative of five experiments. *D*, Human PBTC were loaded with CFSE and incubated with $ATLa_1$ (10 nM) or vehicle for 6 h. Then the cells were incubated over BSA- or anti-CD3 (2 μg/ml)-coated wells as indicated. After 65 h. the cells were collected and analyzed by FACSort. Results are representative of four experiments.

were designed with specific modifications (Fig. 2) to resist ω-oxidation and dehydrogenation and thereby to potentiate their actions (23). A new ATL analog, $ATLa_2$ was prepared and examined for its action. This analog that contains a trifluoro-phenoxyl group at the ω-end was designed building on ATL with the addition of a trifluoro group, which was shown previously to prolong the action of eicosanoids by protection from inactivation (30). LXA_4 and LXB_4 are positional isomers that bind different receptors and in some systems exert different bioactivities (31). To examine the structural requirements for LX- and ATL-evoked inhibition of TNF-α secretion and the significance of metabolic protection, PBMC were exposed to 15-epi-LXA_4, $ATLa_1$, $ATLa_2$, LXB_4, or 5-(*R*/*S*)-methyl-LXB_4 at 0.01–10 nM and then activated by anti-CD3. The results in Fig. 3*A* indicate that $ATLa_1$ inhibits TNF-α secretion from activated PBMC to a similar extent as 15-epi-LXA_4 (87.6 ± 2.9% and 86.3 ± 2.1% at 1 nM, respectively). Hence, the 15-epi-LXA_4-stable analog mimics the activity of the natural compound. In addition, albeit to a slightly lower level, the new trifluoro-containing analog $ATLa_2$ was inhibitory as well (62.3 ± 6.8% at 1 nM; Fig. 3*A*), indicating that the trifluoro group addition maintains the activity of 15-epi-LXA_4. Interestingly, LXB_4 and its stable analog 5-(*R*/*S*)-methyl-LXB_4 also exhibited inhibition of TNF-α secretion from anti-CD3-stimulated PBMC, with a similar concentration range to $ATLa_1$ (76.5 ± 5.3 and 63.5 ± 17.5, respectively at 1 nM; Fig. 3*B*). Thus, ATL and LXB_4 regulate T cell functions in a similar manner.

Expression of ALX by PBTC

LXA_4 and ATL exert their actions by binding to a high-affinity, seven-transmembrane G protein-coupled receptor named ALX/FPRL1, which is expressed in peripheral blood neutrophils and monocytes, as well as in the spleen (12, 32). In Fig. 3, we demonstrated inhibition of TNF-α secretion by both 15-epi-LXA_4 and $ATLa_1$, suggesting that this action is mediated through ALX. To examine whether ALX is expressed in PBTC, we prepared highly enriched T cells (>94%) from human peripheral blood. Total RNA was isolated from the cells and RT-PCR product with an apparent molecular mass of ~1.1 kb was obtained after reaction with specific ALX primers. For the purpose of direct comparison, ALX was also amplified from RNA of human PMN. The resulting PCR product was isolated, sequenced, and found to be identical (24–288 bp) to human ALX cloned from PMN and the monocytic cell line THP-1 (Refs. 27 and 33, GenBank accession no. AF054013). Our results indicate that, albeit to lower levels than those in PMN, ALX mRNA is indeed expressed in PBTC (Fig. 4*A*).

$ATLa_1$ inhibits TNF-α secretion from ALX-transfected human T cells

Since ALX is expressed in PBTC we questioned whether it could mediate $ATLa_1$ actions in T cells. To this end, we examined whether expression of ALX in T cells will lead to inhibition of TNF-α secretion from these cells by $ATLa_1$. We expressed ALX

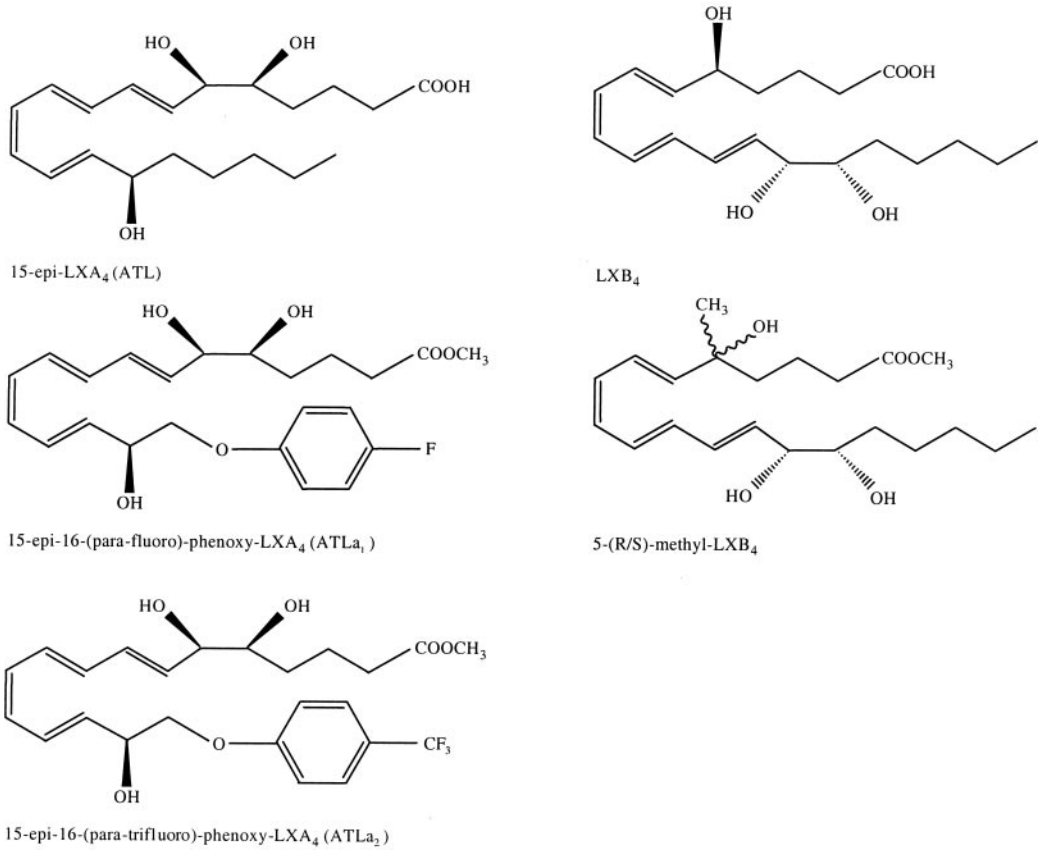

FIGURE 2. Structures of LX-stable analogs. In $ATLa_1$ and $ATLa_2$ modification of 15-epi-LXA_4 was made on C-16 by addition of a fluoro/phenoxyl or trifluorophenoxyl group, respectively, at the para position. LXB_4 is a positional isomer of LXA_4 and in 5-(*R*/*S*)-methyl-LXB_4 the hydrogen at C-5 was replaced by a methyl group as a racemate. Note that the stable analogs were used in the methyl ester form for the experiments.

(J/ALX) or mock (J/mock) cDNA in Jurkat $CD4^+$ T cells. The cells were then exposed to $ATLa_1$ or LXB_4 at 0.001–0.1 nM concentrations and then activated by anti-CD3 plus PMA. TNF-α secretion from J/ALX and J/mock ranged typically around 1.71 $\pm$ 0.1 and 1.3 $\pm$ 0.12 ng/ml, respectively. The results indicate that $ATLa_1$ significantly inhibited TNF-α secretion induced by anti-CD3 plus PMA from J/ALX (Fig. 4*B*). TNF-α secretion from J/mock cells was not reduced but rather increased after $ATLa_1$ treatment (25.5 $\pm$ 5.8 and 46.78 $\pm$ 8.2% increase at 0.01 and 0.1 nM, respectively). Since ALX does not cross-react with LXB_4, no significant inhibition of TNF-α secretion was observed when J/ALX cells were treated by LXB_4. Thus, our findings indicate that treatment of ALX-expressing activated T cells with $ATLa_1$ inhibits TNF-α secretion by these cells.

$ATLa_1$ inhibits ERK activation by anti-CD3 Abs in ALX-expressing T cells

MAP kinases are serine/threonine kinases that serve as key regulators of downstream functions of T cells. ERK belongs to this family and regulates fundamental cellular processes in T cell functioning, such as proliferation and positive or negative selection in thymic development (34). Therefore, we examined whether anti-CD3-induced ERK activity in T cells is regulated by $ATLa_1$. To this end, we treated ALX-transfected Jurkat T cells with $ATLa_1$ (10 nM) or vehicle (0.05% ethanol), and then exposed the cells to immobilized anti-CD3 for 1–5 min. Then the levels of ERK phosphorylation in the cells were determined. The results indicate a significant inhibition of ERK phosphorylation by $ATLa_1$ after 2–5 min (Fig. 5, *A* and *B*). Thus, $ATLa_1$ modulates ERK activity in T cells.

ERK activation is required for the induction of TNF-α secretion

To examine whether inhibition of ERK activation is sufficient to inhibit TNF-α secretion from T cells, PBMC were exposed to either MEK or p38 MAPK inhibitors, namely, U0126 and SB203580 (35, 36), and then activated by anti-CD3. Then the supernatants were collected and evaluated for their TNF-α content. The results in Fig. 5*C* indicate that TNF-α secretion from PBTC was inhibited by U0126 (73.5 $\pm$ 3.9% inhibition), but not by SB203580. Of interest, the aggregation of anti-CD3-activated cells was inhibited by both inhibitors (data not shown), indicating that they were effective at these concentrations. Thus, TNF-α secretion from anti-CD3-stimulated T cells depends on the activation of ERK, but not of p38 MAPK.

Discussion

In the present study, we established a novel function for LX acting on a previously unappreciated cell target, namely, human T cells. Both 15-epi-LXA_4 and LXB_4 inhibited TNF-α secretion from PBMC stimulated by anti-CD3 Abs. We also found that stable analogs of 15-epi-LXA_4 and LXB_4, namely, $ATLa_1$, $ATLa_2$, and 5-(*R*/*S*)-methyl-LXB_4, each share this activity and inhibit TNF-α secretion to a similar extent as their corresponding origins, implying that these analogs could be used for improved in vivo treatment of T cell-mediated inflammation. Most results obtained to date indicate that TNF-α, which is produced by T cells upon stimulation by Ags, is also critical for the establishment of chronic inflammatory disorders such as arthritis, inflammatory bowel disease, and others (20, 37), and therefore serves as a primary target for therapeutic intervention (21, 38). Of interest, TNF-α also plays

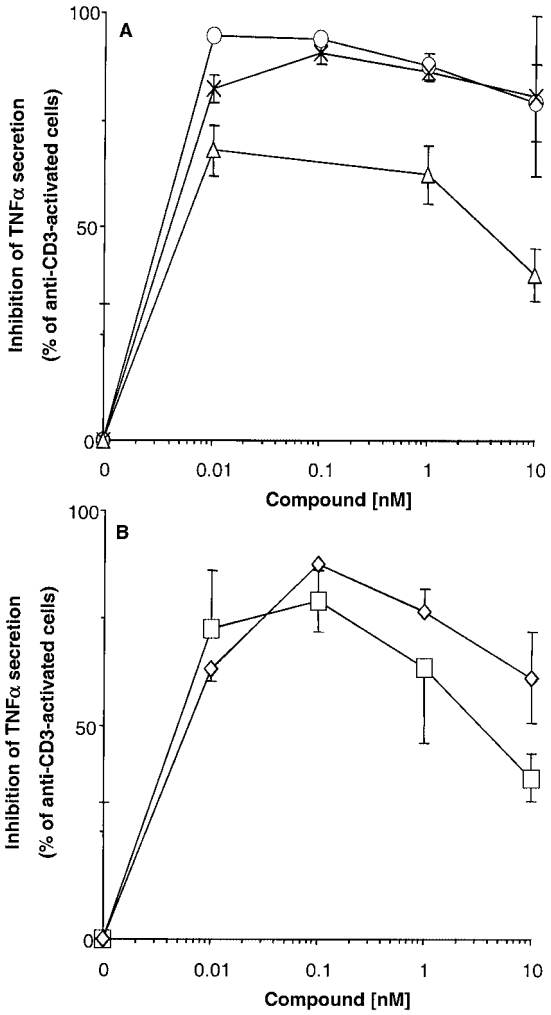

FIGURE 3. Modulation by ATL (*A*) and LXB_4 (*B*) analogs of TNF-α secretion from PBMC after stimulation by anti-CD3 Abs. Human PBMC were incubated with 15-epi-LXA_4 (×, *A*), $ATLa_1$ (○, *A*), $ATLa_2$ (△, *A*), LXB_4 (◇, *B*), or 5-(*R*/*S*)-methyl-LXB_4 (□, *B*) at the indicated concentrations or vehicle for 6 h. Afterward, the cells were incubated in wells that were precoated by anti-CD3 Abs (2 μg/ml). Next, TNF-α levels in the culture medium were determined. All analog treatments were $p < 0.005$ vs vehicle treatment. All $ATLa_2$ vs $ATLa_1$ at the same concentration were $p < 0.005$. Results are the mean ± SD representative of five experiments.

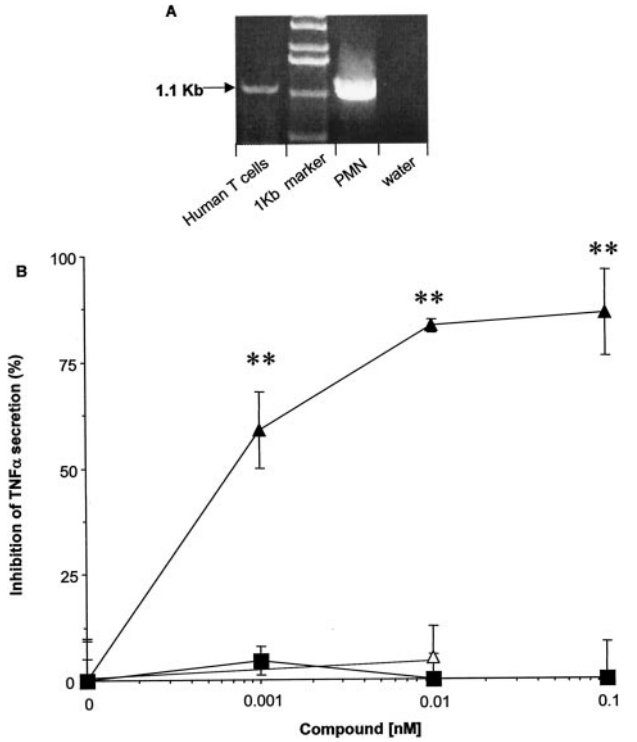

FIGURE 4. *A*, Expression of ALX by PBTC. Total RNA was extracted from human PBTC or PMN and used for RT-PCR analysis to measure ALX expression. Amplified cDNA fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and photographed. Results are representative of three experiments. *B*, Inhibition of TNF-α secretion induced by anti-CD3 plus PMA from Jurkat cells transfected with ALX. ALX (J/ALX, △ and ▲)- or mock (J/mock, ■)-transfected Jurkat cells were incubated with $ATLa_1$ (▲ and ■) or LXB_4 (△) at the indicated concentrations or vehicle, and then incubated over anti-CD3 (2 μg/ml)-coated wells and added with PMA (20 nM). After 16 h at 37°C, the supernatants were collected and their TNF-α content was determined. **, $p < 0.005$ vs vehicle treatment. Results are the mean ± SD representative of three experiments.

a role in the resolution phase of inflammation (18, 39), rendering the regulation of its bioavailability an even more important role than previously appreciated.

LX inhibit TNF-α-evoked functions of PMN and epithelial cells (10, 40). Our results indicate that LX analogs inhibit the production of TNF-α from PBMC as well, establishing a more comprehensive regulatory activity for LX than acknowledged so far, and implying a higher therapeutic potential for these eicosanoids in the treatment of chronic inflammation.

LXA_4 and its epimer 15-epi-LXA_4 (ATL) generate their actions by binding to ALX, a specific G protein-coupled receptor that recognizes ATL as well as short peptides (32, 41). LXB_4 modulates its functions through a different yet unknown receptor (31). To date, ALX is identified by function and/or direct action and is cloned in both human and mouse PMN (12, 33), human monocytes (27), enterocytes (40), and synovial fibroblasts (42). ALX belongs to the chemotactic peptide receptors and shares a high homology with the BLT, but not with prostanoid receptors. We found that 15-epi-LXA_4 and its stable analogs inhibit TNF-α secretion from activated PBMC in similar kinetics, suggesting that these compounds exert their actions through binding to ALX. Consequently, we detected the expression, albeit weak, of ALX in purified human PBTC. This finding suggests that 15-epi-LXA_4 acts directly on T cells through ALX. In addition, we found that only ALX-expressing T cells respond to $ATLa_1$ by inhibition of TNF-α secretion. Of interest, LXB_4 exerts similar actions to $ATLa_1$, although through a different receptor. Together our findings indicate that T cell-expressed ALX mediates $ATLa_1$ generated inhibition of TNF-α secretion from T cells stimulated by TCR-like activation.

LXA_4 modulates the activity of several intracellular signaling elements (43). For example, LXA_4 and ATL regulate transcription of regulatory elements such as the transcriptional corepressor NAB-1 in neutrophils (44) and the suppressor of cytokine signaling 1 and 2 in renal cells in vivo (45). In addition, it was recently demonstrated that LXA_4 induces the activation of ERK and p38 MAPK in renal mesangial cells through different receptors (46). Our findings show that treatment of T cells with $ATLa_1$ inhibits the activation of ERK by anti-CD3 in ALX-transfected T cells (Fig. 5, *A* and *B*) as well as in PBTC (data not shown; $n = 2$). Along these lines, a different LXA_4-stable analog, namely, 15-(*R*/*S*)-methyl-LXA_4, inhibits the phosphorylation of ERK-2 induced by TNF-α in human neutrophils (M. Hachicha and C. N. Serhan, unpublished observations). In addition, we found that specific inhibition of

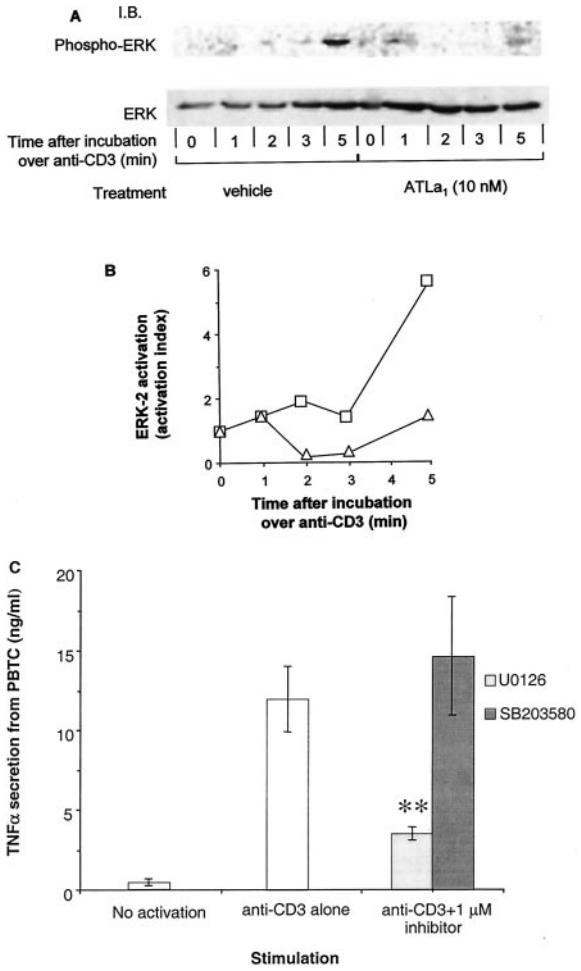

FIGURE 5. Inhibition of anti-CD3-induced phosphorylation of ERK in T cells by $ATLa_1$. *A*, ALX-transfected Jurkat T cells were treated with $ATLa_1$ (10 nM) or vehicle for 6 h at 37°C and then incubated over anti-CD3-coated wells for 1–5 min. Then the cells were placed over ice, added with cold PBS, collected, lysed, and run by SDS-PAGE. The separated proteins were blotted with anti-phospho-ERK Abs. Next, the membranes were stripped and blotted again with an Ab directed against total ERK. *B*, Densitometric analysis was performed for the representative blot and activation index was calculated as indicated in *Materials and Methods*. Different time 0 MD was used for vehicle (□) and $ATLa_1$ (△) treatments. *C*, Inhibition of anti-CD3-induced secretion of TNF-α from human T cells by MEK, but not p38 MAPK inhibitor. Freshly isolated T cells were treated with U0126 or SB203580 (1 μM) and then seeded over anti-CD3 (2 μg/ml)-coated wells and cultured for 65 h. Supernatants were collected and their TNF-α content was determined. **, $p < 0.005$ vs vehicle treatment. Results are the mean ± SD representative of three experiments.

MEK1 and 2 by U0126, leading to ERK inactivation, but not inhibition of p38 MAPK, results in significant inhibition of TNF-α secretion from human T cells. These findings are in accord with previous studies that determined that ERK activation is required for TNF-α secretion from anti-CD3/CD28-activated T cells, although responses to stimulation through CD28 are p38 MAPK dependent (36). Interestingly, a similar impact was observed when 1 nM $ATLa_1$ and 1 μM U0126 were used to block TNF-α secretion, indicating the higher therapeutic potency of $ATLa_1$. Hence, our findings establish an intracellular signaling route which is essential for T cell activation and regulated by LXA_4, suggesting that other TCR-evoked ERK-mediated functions might also be regulated by LX.

Taken together, our present results reveal that both ATL and LXB_4 inhibit TNF-α secretion from T cells activated through their TCR and that ATL action is mediated through ALX, which is expressed by PBTC, and results in inhibition of ERK activation. Moreover, LX-stable analogs retain bioactivity in T cells and thus are valuable tools in elucidating LX and ATL actions on T cells. Thus, LX and ATL are potent endogenous lipid mediators that regulate T cell responses and might be a new therapeutic treatment that will contain chronic inflammation and autoimmune disorders.

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