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Short Communication

Selectivity of Recombinant Human Leukotriene D_4, Leukotriene B_4, and Lipoxin A_4 Receptors with Aspirin-Triggered 15-epi-LXA_4 and Regulation of Vascular and Inflammatory Responses

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Aspirin-triggered lipoxin A_4 (ATL, 15-epi-LXA_4) and leukotriene D_4 (LTD_4) possess opposing vascular actions mediated via receptors distinct from the LXA_4 receptor (ALX) that is involved in leukocyte trafficking. Here, we identified these receptors by nucleotide sequencing and demonstrate that LTD_4 receptor ($CysLT_1$) is induced in human vascular endothelia by interleukin-1β. Recombinant $CysLT_1$ receptor gave stereospecific binding with both [^{3}H]-LTD_4 and a novel labeled mimetic of ATL ([^{3}H]-ATLa) that was displaced with LTD_4 and ATLa ($\sim IC_{50}$ 0.2 to 0.9 nmol/L), but not with a bioinactive ATL isomer. The clinically used $CysLT_1$ receptor antagonist, Singulair, showed a lower rank order for competition with [^{3}H]-ATLa ($IC_{50} \approx 8.3$ nmol/L). In contrast, LTD_4 was an ineffective competitive ligand for recombinant ALX receptor with [^{3}H]-ATLa, and ATLa did not compete for [^{3}H]-LTB_4 binding with recombinant LTB_4 receptor. Endogenous murine $CysLT_1$ receptors also gave specific [^{3}H]-ATLa binding that was displaced with essentially equal affinity by LTD_4 or ATLa. Systemic ATLa proved to be a potent inhibitor (>50%) of $CysLT_1$-mediated vascular leakage in murine skin (200 μg/kg) in addition to its ability to block polymorphonuclear leukocyte recruitment to dorsal air pouch (4 μg/kg). These results indicate that ATL and LTD_4 bind and compete with equal affinity at $CysLT_1$, providing a molecular basis for aspirin-triggered LXs serving as a local damper of both vascular $CysLT_1$ signals as well as ALX receptor-regulated polymorphonuclear leukocyte traffic. ***(Am J Pathol 2001, 158:3–9)***

Leukotrienes (LTs) and lipoxins (LXs) are local mediators formed rapidly within the microenvironment of vascular lumen and at sites of inflammation during cell-cell interactions.[1] In these inflammatory circuits LX counter not only LT bioactions but also their formation.[2] Inflammatory diseases are associated with an increase in specific mediators, their receptors, as well as key pathways such as cyclooxygenase II and lipoxygenase.[2–5] In humans, an array of symptoms are often treated with aspirin (ASA), a lead nonsteroidal anti-inflammatory drug that has beneficial actions that can surpass ASA's well-appreciated ability to inhibit prostaglandin generation in certain clinical settings. These include prevention of cardiovascular diseases as well as decreasing the incidence of lung, colon, and breast cancer.[6] Unlike other nonsteroidal anti-inflammatory drugs that inhibit cyclooxygenase II, ASA also switches the enzyme's activity from prostaglandin endoperoxide synthesis to an R-lipoxygenase that initiates the biosynthesis of endogenous analogues of LX, namely 15-epimeric-LX, also termed aspirin-triggered LX (ATL, 5*S*,6*R*,15*R*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid). These enantiomers of native LX display enhanced anti-leukocyte actions that accompany the chiral switch from S to R at carbon 15 in aspirin-triggered LXA_4.[2] Amplification of this endogenous anti-inflammatory circuit appears to mediate, at least in part, some of ASA's known beneficial actions. LX and ATL mimetics retard inactivation and have potent beneficial actions in acute inflammation as well as in reperfusion injury, providing evidence for novel

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regulatory roles of LX and ATL in leukocyte-mediated events.[2,7,8]

LX and ATL mediate their leukocyte selective actions via their own specific G-protein-coupled receptor, denoted ALX receptor.[2] In addition, LX possess vascular regulatory and smooth muscle actions.[9,10] These unique responses are mediated via recognition sites distinct from the ALX receptor that are shared with cysteinyl-LT (LTD_4/LTC_4) in both vascular[10] and mesangial[11] cells. The slow reacting substances of anaphylaxis (LTC_4, LTD_4, and LTE_4) are of particular interest as they evoke vasculature and smooth muscle responses.[1] Along these lines, bronchospastic activity of LTD_4 is extensively studied as a target for asthma treatment.[12] LTD_4/LTC_4 recognition sites show cell, tissue, and species variation and are broadly classified as $CysLT_1$ or $CysLT_2$ on the basis of rank order with xenobiotic antagonist,[13,14] operational definitions that anteceded molecular cloning and identification of these receptors. Efforts to obtain LTD_4 antagonists initially focused on recognition sites and signal transduction.[13,15] It is of interest then that both LX and ATL functionally antagonize LTD_4/LTC_4-induced up-regulation of P-selectin in endothelial cells[16] and inhalation of LXA_4 antagonizes LTC_4-induced airway obstruction in asthmatics.[17] Thus, both LX and ATL are of interest as mediators because they might act as local endogenous regulators of LTD_4/LTC_4 within the vasculature of lung and kidney[2,8,10,16] as well as control leukocyte trafficking.[2,11]

Recently the first human CysLT receptor and human LTB_4 receptors (BLT) were cloned and identified[14,18,19] using rank order potencies of agonists and antagonists, and a human BLT was overexpressed in transgenic mice.[7] LX and ATL mimetics dramatically inhibit BLT amplified responses in these mice. Because the interactions of recombinant $CysLT_1$ receptor with aspirin-triggered mediators (ie, ATL) have not been established, we sought to identify cysteinyl-LT/LX vascular recognition sites and determine its relationship to the recombinant receptors for LTB_4 and LXA_4. Here, we provide the first direct evidence for a vascular CysLT receptor and its interactions with LXA_4 and aspirin-triggered LXA_4.

Materials and Methods

Receptor Expression

Human umbilical vein endothelial cells (HUVECs) and colonic epithelial cells (T84) were cultured and fresh peripheral blood polymorphonuclear leukocytes (PMN) isolated.[4,16] HUVECs were exposed to interleukin-1β (IL-1β) (1 ng/ml, R&D Systems, Minneapolis, MN) or media alone. Total RNA was isolated (Trizol; Life Technologies, Inc., Grand Island, NY) and reverse transcribed (42°C, 30 minutes) followed by 40 cycles of polymerase chain reaction (PCR) (denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, extension at 72°C for 3 minutes) using specific amino- and carboxyl-terminal primers carrying *Bam*HI and *Xho*I restriction sites, respectively, for $CysLT_1$ receptor ($hCysLT_1$-N: 5′GGCGGATCCATGGA TGAAACAGGA AATCTG-3′ and $hCysLT_1$-C: 5′-CGGCT-CGAGCTATACTTTA CATATTTCTTC-3′). These were designed using reported sequence.[20] Total RNA was analyzed with primers specific for glyceraldehyde-3-phosphate dehydrogenase, PCR products from each cell type were isolated and their complete nucleotide sequences confirmed (BWH DNA Sequencing Facility, Boston, MA).

Cloning, Functional Expression, and Competition Binding

The confirmed full-length $CysLT_1$ receptor cDNA was digested with *Bam*HI and *Xho*I, cloned into pcDNA3 vector carrying a neomycin-resistant gene (Invitrogen Corp., Carlsbad, CA) and transfected into HEK293, CHO, and COS-7 cells using SuperFect (Qiagen Inc., Valencia, CA). Stable transfectants were obtained by selection with G418 sulfate (0.5 mg/ml; Mediatech, Herndon, VA) and expression of $CysLT_1$ receptor was verified by reverse transcriptase (RT)-PCR analysis. [11,12-^{3}H]-15-epi-16-(para-fluoro)-phenoxy-LXA_4 methyl ester ([^{3}H]-ATLa, specific activity ~15 to 30 Ci/mmol; a gift of Dr. H. D. Perez, Berlex Biosciences Inc., Richmond, CA) was prepared with Schering AG (Berlin, Germany) using acetylenic-LXA_4-methyl ester precursor by Dr. Gay (Scherina AG) and further purified and characterized in this laboratory by reverse phase-high pressure liquid chromatography essentially as in Chiang et al.[23] Specific activity and radiolabel purity (>97%) were determined by reverse phase-high pressure liquid radiochromatography equipped with diode array analyses. Retention times and UV chromophore-matched synthetic ATLa. Montelukast (Singulair; Merck and Co., Inc., West Point, PA) was obtained from the Pulmonary Division of Brigham and Women's Hospital. Binding studies were performed essentially as in Sarau et al[14] with 1 nmol/L [14,15,19,20-^{3}H]-LTD_4 (specific activity, ~158 Ci/mmol; Dupont-New England Nuclear, Boston, MA), or [^{3}H]-ATLa and 250 to 350 μg of isolated membrane protein from stable transfectants or rat lungs. Samples were incubated in 250 μl of 10 mmol/L piperazine-N,N′-bis(2-ethanesulfonic acid) (pH 6.5; Sigma Chemical Co., St. Louis, MO), 10 mmol/L $CaCl_2$, 10 mmol/L $MgCl_2$, 10 mmol/L glycine, and 10 mmol/L cysteine for 45 minutes at 25°C as in Sarau et al.[14] Nonspecific binding was determined in the presence of either 100 nmol/L unlabeled LTD_4 or 15-epi-16-(para-fluoro)-phenoxy-LXA_4 methyl ester and routinely accounted for ~50% of total binding. Bound and free [^{3}H]-LTD_4 or [^{3}H]-ATLa was separated by rapid filtration, under vacuum, through Whatman GF/C filters (Fisher, Pittsburgh, PA). Filters were washed three times (5 ml) with ice-cold Tris (10 mmol/L, pH 7.4) and residual [^{3}H]-LTD_4 or [^{3}H]-ATLa retained on the filters was quantitated (Beckman scintillation counter; Beckman-Coulter, Inc., Fullerton, CA). Total and nonspecific binding for [^{3}H]-LTD_4 or [^{3}H]-ATLa was ~4,800 dpm and 2,400 dpm or 3,400 dpm and 1,800 dpm, respectively. HEK293 cells stably transfected with either human recombinant LXA_4 or LTB_4 receptor were prepared for competition binding

with 1 nmol/L of [^{3}H]-ATLa or [5,6,8,9,11,12,14,15-^{3}H]-LTB_4 (specific activity, ~200 Ci/mmol; Dupont-New England Nuclear), respectively.[7,23]

Murine Vascular Leakage and Inflammation

BALB/c male mice (Charles River Laboratories, Wilmington, MA), 24.6 ± 0.5 g, $n = 32$, were anesthetized with pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL) 50 mg/kg i.p. Sterile 0.9% saline (100 μl) containing either EtOH (vehicle), LTD_4 (0.5 μg; Cayman Chemical, Ann Arbor, MI), ATLa (5 μg) alone, or both LTD_4 and MK571, a $CysLT_1$ receptor antagonist (5 μg; Cayman Chemical), or aspirin-triggered LXA_4 analog, were prepared immediately before injection in left tail vein. Mice were given a single bolus intravenous injection and after 90 seconds mice received a second intravenous injection (left tail vein) of sterile saline (100 μl) containing Evan's blue (2%, w/v). Animals were euthanized after 10 minutes and entire external ears were removed for quantitation of punctated vascular leakage. Animals within each experimental group were of the same strain, sex, as well as age, and there were no significant differences in total ear area among mice. Ear sections were placed in 800 μl of formamide and subjected to four cycles of freeze/thaw, and incubated at 50°C for 120 minutes to extract Evan's blue, which was quantitated by monitoring absorbance at 610 nm with a subtraction of nonspecific background (450 to 480 nm). Air pouch experiments were performed as in Hachicha et al[21] using intravenous tail vein injections. Differences between individual data points were analyzed using Student's *t*-test with a two-tailed distribution; values < 0.05 were taken as significant.

Results

We identified vascular LX/cysteinyl-LT sites (Figure 1). RT-PCR with specific $CysLT_1$ receptor primers (see Methods) revealed a single product ~1 kb in mucosal epithelial cells (Figure 1A) also present in leukocytes (not shown), but not apparent in vascular endothelial cells without addition of IL-1β (Figure 1A). Receptor expression and the appearance of new cellular phenotypes induced by inflammatory mediators are now recognized as key regulatory events.[4,5,7] When HUVECs were exposed to IL-1β, a cytokine that induces many features of inflammation,[22] we observed a dramatic time-dependent induction of RNA for $CysLT_1$ receptor (Figure 1A). Complete sequencing of the PCR product (1,014 bp) revealed that the nucleotide sequence amplified from each cell type was identical (not shown) and corresponded to the recently reported[18] human $CysLT_1$ receptor (Figure 1). Comparison of the deduced amino acid sequence of $CysLT_1$ with the two other human receptors for lipoxygenase-derived products, namely LXA_4 (ALX^2) and LTB_4 (BLT^{19}) receptor, showed an overall ~30% homology. ALX and $CysLT_1$ receptor gave highest homology (47%) in the seventh transmembrane segment that is associated with ligand recognition[23] for eicosanoid receptors,

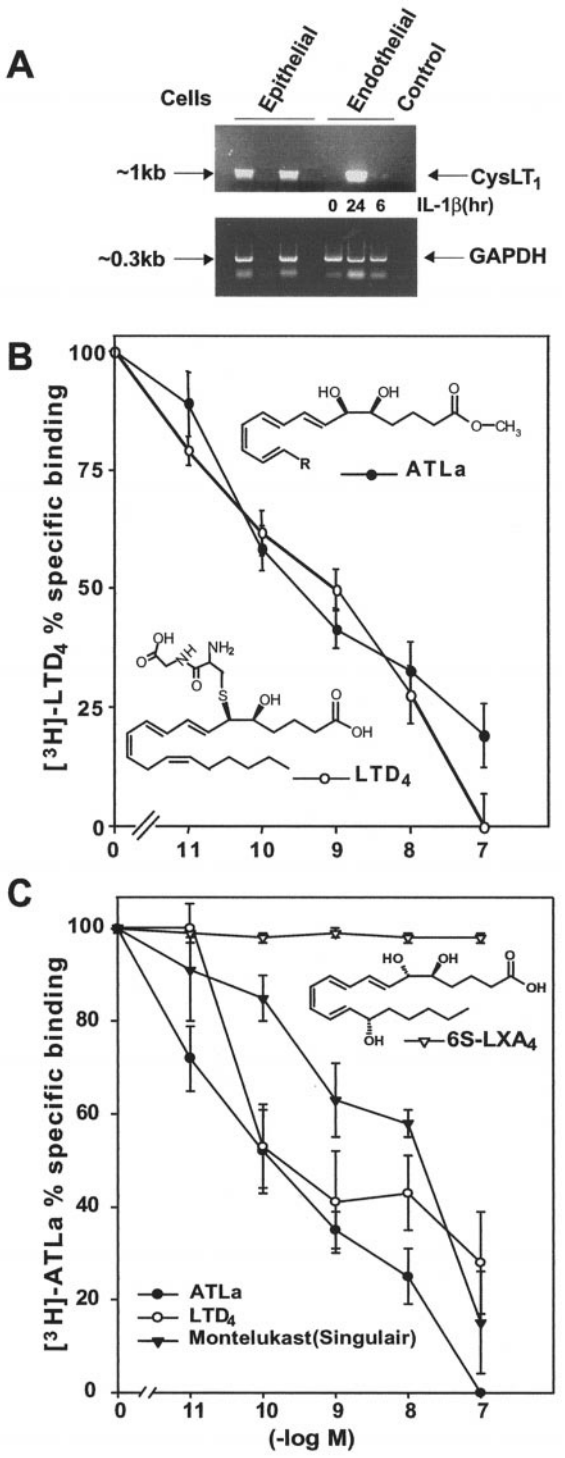

Figure 1. Identification of human vascular endothelial-derived $CysLT_1$ receptor. **A:** $CysLT_1$ receptor RNA expression in mucosal epithelial cells and IL-1β induction in HUVECs. RT-PCR results (see Methods) are from two separate T84 cell RNA samples in parallel lanes ($n = 4$), a representative for HUVECs exposed to IL-1β (6 or 24 hours) or media alone ($n = 3$), and PCR control (without RNA, molecular size of products is indicated by **arrow**). **B:** Competition for specific [^{3}H]-LTD_4 (1 nmol/L) binding with increasing concentrations of LTD_4 or ATLa in isolated membrane preparations from $CysLT_1$ receptor stable transfectants (COS-7 cells, $n = 3$ with duplicates). Structures of the $CysLT_1$ receptor competitors are shown in **inset**. **C:** Competition for [11,12-^{3}H]-ATLa (1 nmol/L)-specific binding with increasing concentrations of ATLa, the specific $CysLT_1$ receptor antagonist montelukast or the bioinactive 6*S*-LXA_4 (**inset**) in isolated membrane preparations from $CysLT_1$ receptor stable transfectants (COS-7 cells, $n = 3$ with duplicates).

whereas the first cytoplasmic loop that is associated with G-protein coupling shows no homology between ALX and $CysLT_1$ receptor but is highly conserved (56%) among BLT and ALX receptor (not shown). These findings provide the first direct evidence for a cytokine-inducible endothelial cysteinyl-LT receptor, namely $CysLT_1$.

LTD_4 and LXA_4 compete for specific binding at shared receptors in human vascular endothelial cells.[11] However, the rank order of cysteinyl LTs in inducing surface expression of P-selectin, a well-characterized vascular action of cysteinyl-LT, and the inability of selective $CysLT_1$ receptor antagonists to inhibit this response suggest that these endothelial receptors are likely distinct from $CysLT_1$.[16,24,25] Although the $CysLT_1$ receptor was identified and cloned by other investigators,[14,18] it was not formally cloned or identified from human vascular endothelial cells. The available evidence that this receptor was indeed a major endothelial receptor remained incomplete. Thus it was of interest that $CysLT_1$ receptor RNA expression was only detected in vascular endothelial cells that were exposed to IL-1β (Figure 1). To directly test whether LTD_4 and LXA_4 both interact at $CysLT_1$ we cloned and expressed the vascular endothelial $CysLT_1$ receptor as stable transfectants in COS-7 cells. Of the endogenous ligands reported in competition binding and functional assays with either recombinant human leukocyte or spleen $CysLT_1$ receptor, LTD_4 is the most potent.[14,18] Figure 1 reports results of competition binding with [^{3}H]-LTD_4 and recombinant $CysLT_1$ receptor. Binding with recombinant human $CysLT_1$ receptor was specific and high affinity with COS-7 cell stable transfectants. Isolated COS-7 cell membrane preparations gave specific binding (apparent IC_{50} of ~0.7 nmol/L for unlabeled LTD_4; Figure 1B), a value consistent with those reported.[18] To determine whether ATL interacts with $CysLT_1$, a stable analog of aspirin-triggered LXA_4 (ATLa) with anti-inflammatory activities[2] was assessed for its ability to displace [^{3}H]-LTD_4. Unexpectedly, ATLa that carries a 15-epi configuration displaced [^{3}H]-LTD_4 with essentially equivalent affinity, IC_{50} of ~0.7 nmol/L, when directly compared to LTD_4. Virtually identical results were obtained with another LXA_4 and ATL mimetic 15R/S-methyl LXA_4 (not shown). Together, these findings provide the first direct evidence that ATLa competes for specific LTD_4 binding at recombinant human $CysLT_1$ found on endothelial cells.

Next, a new tritiated ATLa was prepared to directly assess its affinity for $CysLT_1$ receptor (see Methods). Both ATLa and LTD_4 competed with high affinity for specific [^{3}H]-ATLa binding at recombinant endothelial $CysLT_1$ receptor (Figure 1C) with an apparent IC_{50} of ~0.1 nmol/L and ~0.9 nmol/L, respectively. For the purpose of direct comparison, the well-defined and reported selective $CysLT_1$ antagonist[18] montelukast (Singulair, MK476) was assessed and gave a lower rank order potency for displacing [^{3}H]-ATLa than either LTD_4 or ATLa with an apparent IC_{50} of ~8.3 nmol/L (Figure 1C). To determine whether the $CysLT_1$ receptor recognition of aspirin-triggered LXA_4 was stereoselective, we examined a biologically inactive analog of both LXA_4 and aspirin-triggered LXA_4,[10] namely 6*S*-LXA_4 (5*S*,6*S*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) (see Figure 1C). This LX isomer (0.01 to 100 nmol/L) did not displace [^{3}H]-ATLa, indicating that the *rectus* chirality at carbon 6, a motif shared by both LTD_4 and ATLa, is essential for $CysLT_1$ receptor recognition. Neither specific [^{3}H]-LTD_4 nor [^{3}H]-ATLa binding was observed with wild-type COS-7 cells. Together these findings indicate that ATLa and LTD_4 directly bind to the $CysLT_1$ receptor with equal affinity.

To address ATLa actions with the two other recombinant human receptors, BLT and ALX, competition binding was performed with HEK293 cells stably expressing each of the respective receptors. [^{3}H]-ATLa specifically bound to the ALX receptor with high affinity (Figure 2A), and both unlabeled ATLa and LXA_4 competed for binding with approximately equal affinity with an apparent IC_{50} of ~0.5 nmol/L and ~0.2 nmol/L, respectively. In contrast, LTD_4 was a less effective ligand for the LXA_4 receptor because its IC_{50} for [^{3}H]-ATLa displacement was greater than 3 log orders more than that of the homoligand ATLa. Values are consistent with our earlier findings reported with LTD_4 and the ALX receptor.[26] Of interest, MK571, a specific $CysLT_1$ receptor antagonist,[18] competed for specific [^{3}H]-ATLa binding at ALX receptor with equal affinity (IC_{50} ~ 0.3 nmol/L) compared to the homoligand ATLa and LXA_4 (Figure 2A). For direct comparison, [^{3}H]-LTB_4 specifically bound to BLT receptor with an apparent IC_{50} of ~5.2 nmol/L for its homoligand LTB_4 (Figure 2B, inset), whereas ATLa did not compete at physiologically relevant concentrations (0.1 to 1,000 nmol/L). These results indicate that LXA_4 receptor recognizes ATLa and LXA_4 with essentially equal affinity but that ATLa does not act selectively at LTB_4 receptor. Moreover, structural motifs shared by aspirin-triggered LXA_4 and LTD_4 that are required for $CysLT_1$ receptor binding are alone not sufficient for ligand recognition at LXA_4 receptor.

To determine whether the rank order potency of ATLa in competition binding with recombinant receptor was also a feature for the endogenous $CysLT_1$, we prepared isolated murine lung membranes, as they contain pharmacologically defined rat $CysLT_1$ receptors.[27] [^{3}H]-ATLa (Figure 3A, inset) gave specific binding with lung, and both ATLa and LTD_4 competed with approximately equal affinity, giving an apparent IC_{50} of ~0.2 nmol/L and ~0.8 nmol/L, respectively.

LXA_4 acts as an antagonist and/or partial agonist at pharmacologically defined LTD_4/LTC_4 receptors in a tissue-specific manner[2,9–11] as well as blocks PMN-mediated injury[7] and inflammation.[11,21] To investigate the impact of ATLa on LTD_4-mediated vascular events, we examined systemic vascular leakage as a fundamental action of slow reacting substances.[1] Systemic LTD_4 stimulated rapid (T_0 to 10 minutes) vascular leakage visible in mouse ears (Figure 3B). In contrast, mice treated with the vehicle alone did not show significant vascular leakage in this tissue. LTD_4-stimulated vascular leakage was dose-dependent (not shown) and inhibited (95 ± 3%, $P < 0.02$) by the specific $CysLT_1$ receptor antagonist MK571[18] at a dose of 200 μg/kg. Systemic LTB_4 did not induce vascular leakage in mouse ear tissue within this time interval (n = 3, not shown), whereas LTB_4 as a topical agent

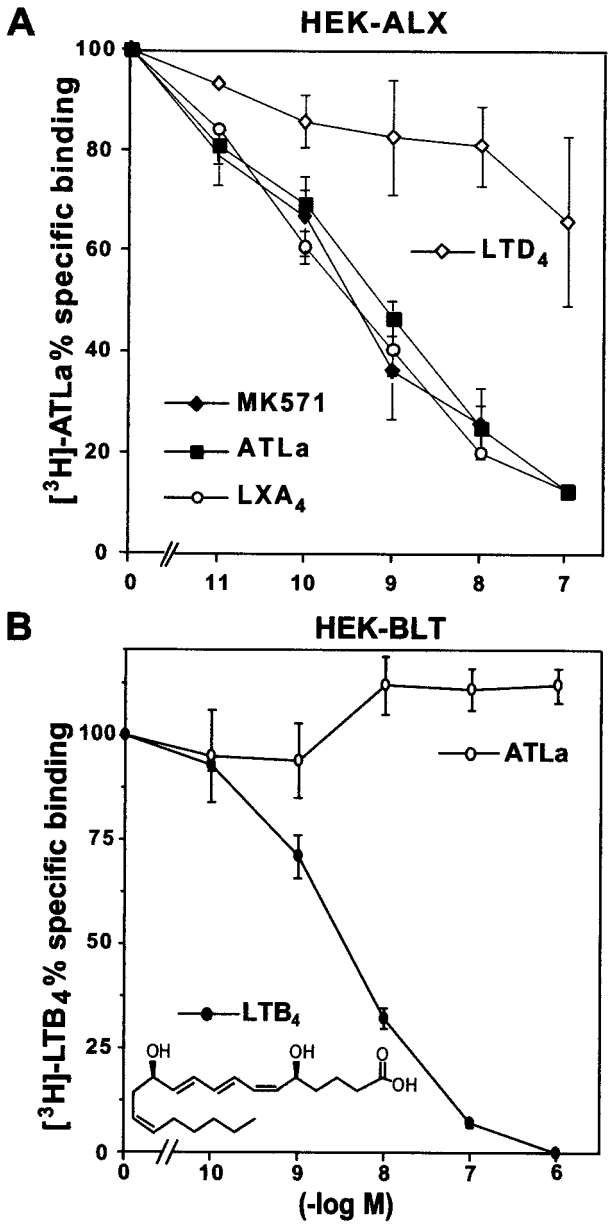

Figure 2. Selectivity of recombinant human LXA_4 and LTB_4 receptor. **A:** Competition for [11,12-^{3}H]-ATLa-specific binding (1 nmol/L) with increasing concentrations of ATLa, LXA_4, or the specific $CysLT_1$ receptor antagonist MK571 in intact cells of ALX receptor stable transfectants (HEK293 cells, n = 3 with duplicates). **B:** Competition for [^{3}H]-LTB_4 (1 nmol/L)-specific binding with ATLa or LTB_4 (**inset**) in intact cells of BLT receptor stable transfectants (HEK293 cells, n = 3 with duplicates).

stimulates PMN recruitment and subsequent PMN-dependent vascular leakage at much longer time intervals (eg, 8 to 48 hours) as in Takano et al.[11] When administered alone ATLa did not evoke vascular leakage, but clearly ATLa treatment inhibited vascular leakage (53 ± 9% at t = 10 minutes, $P < 0.02$ when compared to LTD_4 leakage) at intravenous-administered doses as low as 0.2 mg/kg. For purposes of direct comparison, ATLa was given intravenously (tail vein, 4 μg/kg) and by 4 hours inhibited TNF-α-induced PMN infiltration by 56 ± 10%, $P < 0.02$ into the dorsal air pouch (Figure 3B). These are consistent with earlier findings and at this time interval are held to be mediated by ALX receptors.[21]

Discussion

Self-resolution of acute inflammation is an important component of host defense and homeostasis. A class of small endogenous molecules, namely LX and their aspirin-triggered epimeric forms (ATL), seem to play key roles in promoting and/or maintaining self-resolution by regulating leukocyte trafficking and PMN-endothelial and PMN-epithelial interactions.[2,4,28] LXs and ATL are local-acting lipid mediators that not only inhibit the formation of pro-inflammatory signals such as LT, cytokines (IL-1β), and chemokines (IL-8, MCP-2)[2,4,21] but also block the *in vivo* actions of these signals in that ATL inhibits LT-, cytokine-, as well as chemokine-stimulated leukocyte recruitment.[7,11,21] Results obtained in the present report with recombinant human endothelial $CysLT_1$ (Figures 1 and 2) as well as murine $CysLT_1$ receptor (Figure 3) provide the first direct evidence *in vivo* and *in vitro* for a novel vascular site of action and role for LX and aspirin-triggered LX as endogenous receptor level antagonists of LTD_4.

The magnitude and duration of inflammation as well as the host's response to reperfusion injury are directly dependent on the presence, abundance, and activation of specific cellular receptors.[7] Here, we found that IL-1β, a primary pro-inflammatory cytokine,[22] induces RNA expression for $CysLT_1$ receptor in vascular endothelial cells (Figure 1), suggesting a potentially novel endothelial phenotype and/or role for this receptor in activated vascular tissue. Cytokine regulation of eicosanoid receptors represents important regulatory steps in inflammatory diseases, as both immune function in colonic mucosa and asthmatic responses in OVA-induced asthma are associated with induction of epithelial LXA_4 receptor[4] and prostaglandin D_2 receptor.[5] Thus, it is noteworthy that $CysLT_1$ receptor transcripts were also found in human colonic epithelial cells (Figure 1), where a bioaction for cysteinyl-LT has not yet been firmly established.

In view of the well-appreciated roles of cysteinyl-LT,[1] it is of interest that mimetics of endogenous aspirin-triggered LXA_4 specifically bind to $CysLT_1$ receptor with apparent equal affinity (Figures 1–3) when directly compared to its reported homoligand LTD_4.[14,18] Cysteinyl-LT, LX, and ATL are generated within the vessel lumen during cell-cell interactions, for example during leukocyte interaction with platelets, or with inflamed endothelial or mucosal epithelial tissue.[2,4] Thus these potential local-acting lipid mediators can have access to shared recognition sites (ie, $CysLT_1$ receptor) on leukocytes, endothelial, epithelial, and smooth muscle cells (Figure 1).[14,18] In addition, ATL is a regulator of leukocyte trafficking by acting at the LXA_4 receptor (Figure 2A). These findings with labeled analog and ALX receptor are consistent with earlier results obtained with [^{3}H]-LXA_4 for both human and murine LXA_4 receptors.[11,23] ATLa's potent inhibition of $CysLT_1$ receptor mediated vascular leakage documented here (Figure 3B) strongly indicates that LXA_4 and ATLa are also functionally relevant $CysLT_1$ antagonists *in*

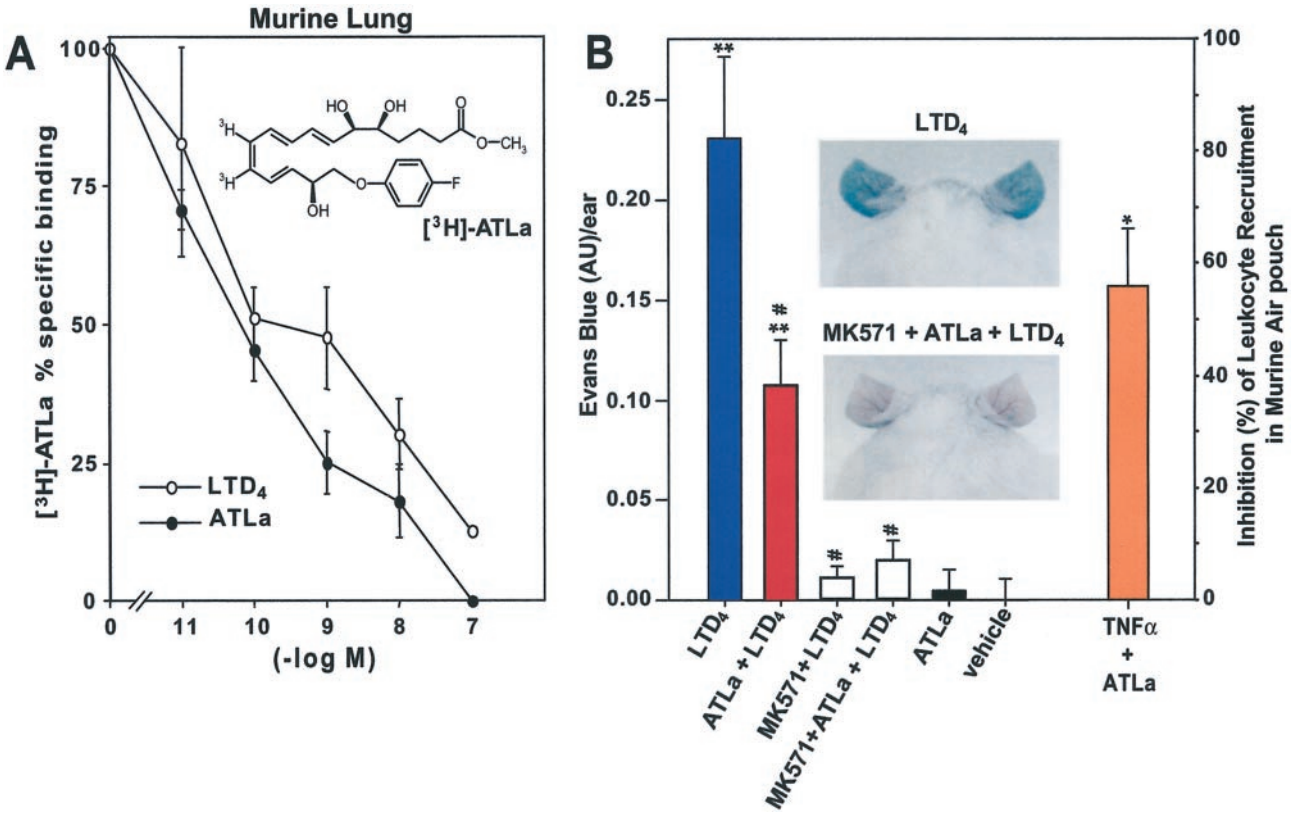

Figure 3. ATLa competes for specific binding at murine $CysLT_1$ receptor as well as inhibits both vascular leakage and PMN trafficking. **A:** Competition for [11,12-^{3}H]-ATLa (1 nmol/L)-specific binding with increasing concentrations of LTD_4 or ATLa in isolated membrane preparations from rat lungs (n = 3 with duplicates). **B:** Inhibition of vascular leakage and leukocyte trafficking. Mice were injected intravenously (left tail vein) with LTD_4 (0.5 μg), ATLa (5 μg), or a single combined injection of LTD_4 (0.5 μg) and ATLa (5 μg) and/or a $CysLT_1$ antagonist (MK571, 5 μg). A second injection of Evans Blue was given 90 seconds after treatment (see Methods). Results represent the mean (n = 3 to 8 with duplicates) above vehicle. #, $P < 0.02$ compared to LTD_4 treatment alone; **, $P < 0.01$ compared to vehicle alone. **Top inset:** LTD_4 (0.5 μg) stimulated vascular leakage (10 minutes after injection) and **bottom inset:** Inhibition with a combined dose of antagonists (ie, ATLa and MK571, each at 5 μg). Right ordinate: TNF-α (20 ng) was injected locally into 6-day dorsal air pouch and ATLa (100 ng) or vehicle injected intravenously (left tail vein) and infiltrated leukocytes quantitated at 4 hours after treatment (n = 3).

vivo and provide evidence for a novel fundamentally protective mechanism that may be in place to dampen overt cysteinyl-LT-generated signals for leakage. Such protective pathway(s) can be evoked and amplified by aspirin, which triggers ATL generation via transcellular biosynthesis between neutrophils and inflamed endothelial cells that possess up-regulated and acetylated cyclooxygenase II[2] at sites of vascular inflammation (Figure 1C). Taken together, these tools, namely mimetics of LX and novel aspirin-triggered lipid mediators, because of their prolonged duration of action *in vivo*, can help to define local counter-regulatory signals acting within the microenvironment that are of interest in inflammation, resolution, and vascular diseases. LX and ATL regulate several components of interest in human disease. For example, they activate ALX receptor that 1) inhibits expression of pro-inflammatory signals[2,4,21]; 2) regulates leukocyte trafficking and sequestration[7,8]; as well as 3) act directly as $CysLT_1$ receptor antagonists established for the first time at the molecular level in the present experiments. In view of these findings, endogenous lipid mediators (eg, LX and ATL) could provide new avenues or alternative approaches to controlling both vascular inflammatory disorders as well as pathophysiological vascular events involving elevated levels of LTD_4.

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