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The Lipoxin Receptor ALX: Potent Ligand-Specific and Stereoselective Actions in Vivo

NAN CHIANG,[1] CHARLES N. SERHAN, SVEN-ERIK DAHLÉN, JEFFREY M. DRAZEN, DOUGLAS W. P. HAY, G. ENRICO ROVATI, TAKAO SHIMIZU, TAKEHIKO YOKOMIZO, AND CHARLES BRINK

The Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine (N.C., C.N.S.), and Department of Medicine (J.M.D.), Brigham and Women's Hospital/Harvard Medical School, Boston, Massachusetts; Department of Oral Medicine, Infection, and Immunity (C.N.S.), Harvard School of Dental Medicine, Boston, Massachusetts; Unit for Experimental Asthma and Allergy, The National Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden (S.-E.D.); GlaxoSmithKline, King of Prussia, Pennsylvania (D.W.P.H.); Division of Molecular Pharmacology, Pharmacological Sciences, Milan, Italy (G.E.R.); Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Tokyo, Tokyo, Japan (T.S.); Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, Fu Kuoka, Japan (T.Y.); and Institut National de la Santé et de la Recherche Médicale U698, Hôpital Xavier Bichat secteur Claude Bernard, Paris, France (C.B.)

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Address correspondence to: Dr. Charles Brink, Researcher (CNRS CR-1), INSERM U698, Hôpital Xavier Bichat secteur Claude Bernard, 46, rue Henri Huchard, 75877 Paris cedex 18, France. E-mail: charlesbrink@hotmail.com
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***Abstract*——Lipoxins (LXs) and aspirin-triggered LX (ATL) are trihydroxytetraene-containing eicosanoids generated from arachidonic acid that are distinct in structure, formation, and function from the many other proinflammatory lipid-derived mediators. These endogenous eicosanoids have now emerged as founding members of the first class of lipid/chemical mediators involved in the resolution of the inflammatory response. Lipoxin A_4 (LXA_4), ATL, and their metabolic stable analogs elicit cellular responses and regulate leukocyte trafficking in vivo by activating the specific receptor, ALX. ALX was the first receptor cloned and identified as a G protein-coupled receptor (GPCR) for lipoxygenase-derived eicosanoids with demonstrated cell type-specific signaling pathways. ALX at the level of DNA has sequence homology to the *N*-formylpeptide receptor and as an orphan GPCR was initially referred to as the *N*-formylpeptide receptor-like 1. Although LXA_4 is the endogenous potent ligand for ALX activation, a number of peptides can also activate this receptor to stimulate calcium mobilization and chemotaxis in vitro. In contrast with LXA_4, the counterparts of many of these peptides in vivo remain to be established. The purpose of this review is to highlight the molecular characterization of the ALX receptor and provide an overview of the ALX-LXA_4 axis responsible for anti-inflammatory and proresolving signals in vivo. The information in this review provides further support for the initial nomenclature proposition for this GPCR as ALX.**

I. Introduction

The accumulation and activation of leukocytes are the hallmarks of most inflammatory disorders. Many of the eicosanoids derived from arachidonic acid (AA[2]), including prostaglandins (PGs) and leukotrienes (LTs), play important roles as local mediators exerting a wide range of actions relevant in immune hypersen-

[2] Abbreviations: AA, arachidonic acid; PG, prostaglandin; LT, leukotriene; LO, lipoxygenase; LXA_4, lipoxin A_4 (5*S*, 6*R*, 15*S*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid); LXB_4, lipoxin B_4 (5*S*, 14*R*, and 15*S*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid); COX, cyclooxygenase; ATL, aspirin-triggered lipoxin A_4 (15-epi-LXA_4; 5*S*, 6*R*, 15*R*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid); ATLa, aspirin-triggered lipoxin stable analog(s); 15*R*-HETE, 15*R*-hydroxyl-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; GPCR, G protein-coupled receptor; ALX, lipoxin A_4 receptor; PMN, polymorphonuclear neutrophil; 15*S*-HETE, 15*S*-hydroxyl-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; NF-κB, nuclear factor-κB; FPRL1, formyl peptide receptor-like 1; FPR, formyl peptide receptor; CHO, Chinese hamster ovary; PTX, pertussis toxin; kb, kilobase(s); BLT, leukotriene B_4 receptor; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; TNF, tumor necrosis factor; TM, transmembrane domain; MHC, major histocompatability complex; RvE1, resolvin E1; HEK, human embryonic kidney; HIV, human immunodeficiency virus; uPAR, urokinase-type plasminogen activator receptor; ANXA1, annexin 1; 15-epi-LXB_4, 15-epimeric-lipoxin B_4 (5*S*, 14*R*, and 15*R*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid); IL, interleukin; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; ALI, acute lung injury; CTGF, connective tissue growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated protein kinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; AP-1, activator protein-1; CysLT, cysteinyl-leukotriene; HUVEC, human umbilical vein endothelial cell; SKF104353, pobilukast; PDGF, platelet-derived growth factor; AhR, Ah receptor; VEGF, vascular endothelial growth factor.

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sitivity and inflammation (Samuelsson et al., 1987). However, recent observations indicate that other agents derived from the lipoxygenase (LO) pathways are formed and play a key role in initiating the resolution of acute inflammation. This phenomenon is an active process that is governed by specific lipid mediators and involves a series of well-orchestrated temporal events (Serhan et al., 2004; Bannenberg et al., 2005). Thus, potent locally released mediators serve as checkpoint controllers of inflammation (reviewed in Nathan, 2002). Within this framework, lipoxin A_4 (LXA_4) is generated endogenously and evokes protective actions in a range of physiologic and pathophysiologic processes (Chiang et al., 2005). Interestingly, aspirin impinges on this process. In addition to the well-appreciated ability of aspirin to inhibit PGs (Vane, 1982), aspirin also acetylates cyclooxygenase (COX)-2, triggering the formation of a 15-epimeric form of lipoxins, termed aspirin-triggered LXA_4 (ATL) (Clària and Serhan, 1995). These eicosanoids (i.e., LXA_4 and ATL) with a unique trihydroxytetraene structure (Fig. 1) function as "stop signals" in inflammation and actively participate in dampening host responses to bring the inflammation to a close, namely, resolution.

LXA_4 and ATL elicit the multicellular responses via a specific G protein-coupled receptor (GPCR) termed ALX (Table 1) that has been identified in human (Fiore et al., 1994), mouse (Takano et al., 1997), and rat (Chiang et al., 2003) tissues. ALX also has the ability to interact in vitro with a wide panel of small peptides/proteins that give different signaling responses than either LXA_4 or ATL, indicating that ALX is capable of serving as a stereoselective yet multirecognition receptor in immune responses. In this nomenclature update, results concerning activation of the ALX receptor are reviewed with respect to pharmacology, molecular biology, ligand specificity, and signal transduction. In addition, experimental evidence in vitro and results derived from animal models that provide pertinent information demonstrating that LXA_4 is the principal endogenous and high-affinity agonist for this receptor during inflammation and its resolution are highlighted. These results offer considerable support for the official nomenclature of this receptor, namely, ALX as proposed earlier (Fiore et al., 1994) and reviewed in Brink et al. (2003).

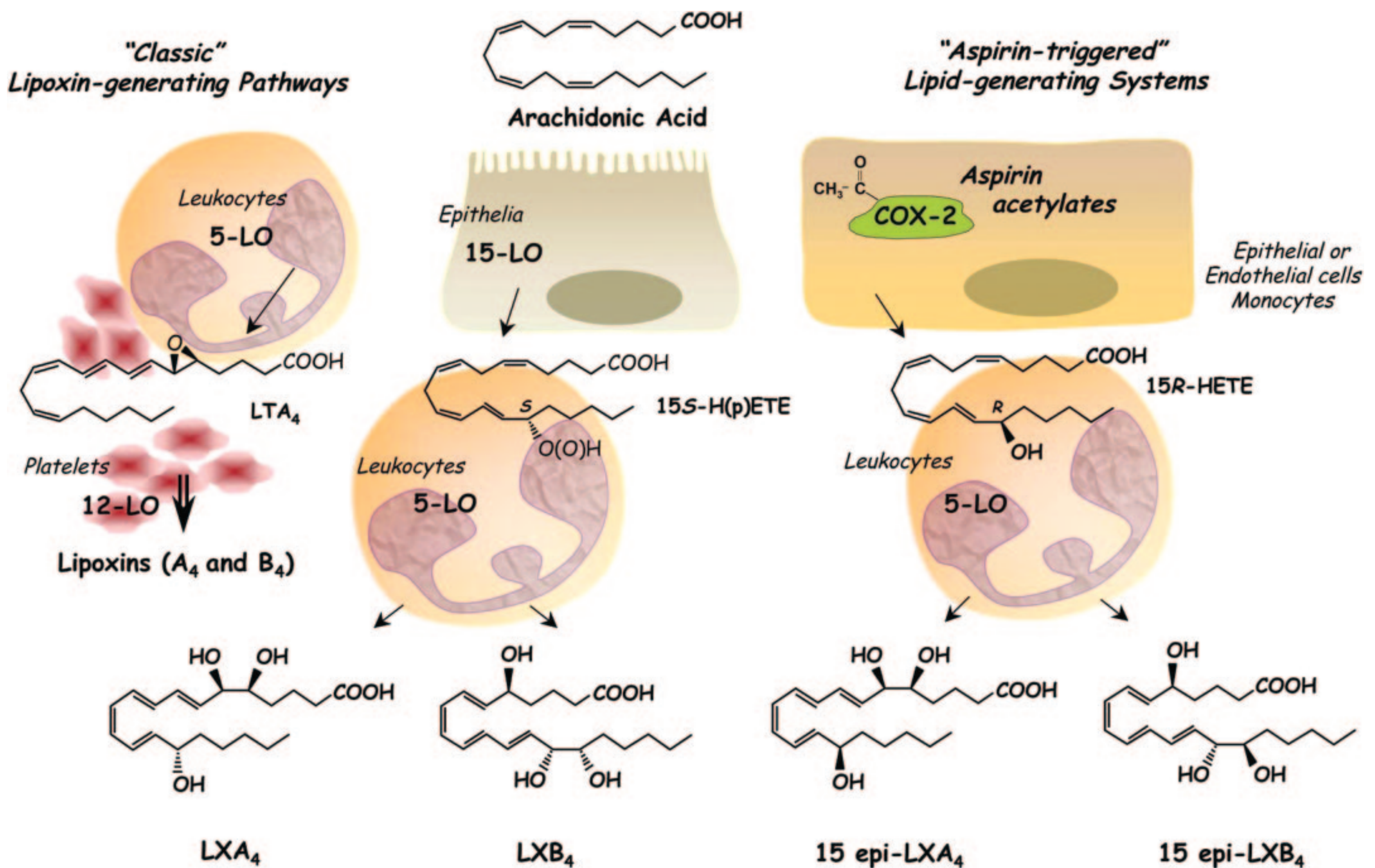

FIG. 1. De novo and aspirin-triggered lipoxin-generating pathways. During cell-cell interactions, lipoxin production can be amplified by transcellular biosynthesis via the interactions of two or more cell types. Two main classic lipoxin-generating pathways via LO interactions seem to be used in human cells and tissues (right side) (Romano and Serhan, 1992; Levy et al., 1993). The aspirin-triggered lipid-generating pathway is initiated when COX-2 is up-regulated and irreversibly acetylated by aspirin. The acetylated COX-2 remains catalytically active, but the enzyme activity switches from generating a prostaglandin intermediate to producing 15*R*-HETE, the precursor of 15-epi-LXs (ATL) (left side) (Clària et al., 1995).

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TABLE 1
The human lipoxin receptor ALX

Name	ALX
Alternate names	FPRL1, FPRH1, FPR2, RFP, HM63
Accession numbers	U81501, AF054013, X63819, M84562, D10922, M88107, M76672
Gene/chromosome	19q
Amino acid composition	351
Selective lipid ligands	LXA_4, 15-epi-LXA_4, ATLa1, ATLa2 (K_d = ~1.7 nM)
Partial competitors	LTC_4, LTD_4 (IC_{50} = ~50 nM), fMLF, 15-deoxy-LXA_4 (IC_{50} = >400 nM)
Receptor expression	Cells: PMN, monocytes, activated T cells, enterocytes, synovial fibroblasts Organs: spleen, lung with lesser amounts in heart, placenta, and liver

II. Production of Lipoxins

A. Transcellular Biosynthetic Pathways

Two major routes of lipoxin (LX) biosynthesis in human cell types have been established (Fig. 1). One pathway involves peripheral blood platelet-leukocyte interactions. Human platelets do not produce LX on their own but become a major source of LX when platelet-PMN adhesion occurs. The leukocyte 5-LO converts AA to the epoxide product LTA_4, which is then released and further transformed by adherent platelets to LXA_4 via the LX synthase activity of human 12-LO (Romano and Serhan, 1992). A second biosynthetic route is initiated at the mucosal surfaces (Serhan, 1997) by 15-LO that inserts molecular oxygen into AA at the carbon 15 position to produce 15*S*-hydroxyleicosatetraenoic acid (15*S*-HETE); this latter metabolite is rapidly taken up by PMNs and is converted subsequently via 5-LO to LXs. This event not only leads to LX biosynthesis but also reduces LT formation. LXA_4 generated by these two pathways carries its C-15 hydroxyl group mainly in the *S*-configuration, which is inserted by LO-based mechanisms. LXB_4 is a positional isomer of LXA_4 (Fig. 1), carrying alcohol groups at the carbon 5*S*, 14*R*, and 15*S* positions [5*S*, 14*R*, and 15*S*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid], instead of the carbon 5*S*, 6*R*, and 15*S* positions present in LXA_4 (5*S*,6*R*,15*S*-trihydroxyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid).

B. Formation of Lipoxin A_4 in Animals and in Humans

LXA_4 is produced in vivo (reviewed in Serhan, 1997) during the course of inflammation as reported in an experimental immune complex glomerulonephritis model (Munger et al., 1999) and in pleural exudate upon allergen challenge in rats (Bandeira-Melo et al., 2000a). In addition, endogenous LXA_4 is produced in ischemic lungs and elevated by reperfusion during hind limb ischemia reperfusion (Chiang et al., 1999). More recently, LXA_4 was shown to be generated during microbial infections, for example, as in a *Toxoplasma gondii*-exposed murine model (Aliberti et al., 2002a,b) and in mouse corneas (Gronert et al., 2005). Of interest, *T. gondii* carries a 15-LO that enhances LXA_4 levels, probably as a mechanism to suppress the host defense (Bannenberg et al., 2004) (see below). Alterations in local LXA_4 levels seem to be linked to the pathophysiology of several human diseases. For example, LXA_4 production is upregulated in human subjects with localized juvenile periodontitis (Pouliot et al., 2000). In addition, LXA_4 formation is established in aspirin-tolerant and aspirin-intolerant asthmatics (Bonnans et al., 2002). Along these lines, a recent report demonstrated that LXA_4 levels in airway fluids are significantly suppressed in patients with cystic fibrosis compared with patients with other inflammatory lung conditions (Karp et al., 2004).

C. Structure-Activity Relationship of Lipoxin A_4

Each action of LXA_4 has proven to be stereoselective in that changes in potencies are associated with double bond isomerization and alcohol chirality (*R* or *S*) as well as dehydrogenation of alcohols and reduction of double bonds (Fig. 2). For example, 15-deoxy-LXA_4 fails to inhibit PMN transmigration and adhesion (Serhan et al., 1995). Furthermore, LXA_4 is enzymatically inactivated by conversion to both 15-oxo-LXA_4 and 13,14-dihydro-LXA_4. These further metabolites are essentially biologically inactive with respect to inhibiting superoxide anion generation and PMN transmigration, which are the key features of LXA_4 (Clish et al., 2000). These pharmacophores for the anti-inflammatory action of LXs (i.e., 15-hydroxyl group and 13,14-double bond) are also required for their interaction with the specific cell surface receptor, ALX. Along these lines, the biologically inactive products, including 15-oxo-LXA_4, 15-deoxy-LXA_4, and 13,14-dihydro-LXA_4, do not bind to ALX, contrasting with the active ligands [e.g., 15*R*- (or 15-epi-) and the native 15*S*-LXA_4] that exhibit stereospecific receptor binding to ALX (Takano et al., 1997). Furthermore, the methyl ester of LXA_4 is a partial ALX antagonist and does not effectively regulate NF-κB activity in vitro (Fierro et al., 2003). In addition, LXA_4 methyl ester markedly blocks PMN transmigration across epithelial or endothelial cells but is less potent in inhibiting PMN chemotaxis. These observations highlight the fact that LXA_4 methyl ester is rapidly hydrolyzed to free acid in vivo and/or in the presence of PMN and other cell types such as epithelial cells that possess the esterase activity. These results suggest that pharmacologic additions of LXA_4 and/or LX analogs as carboxy methyl esters are proligands or prodrugs that require conversion to their corresponding free acids to evoke ALX-mediated biological actions, including signal transduction, phosphorylation, and gene regulation (see below).

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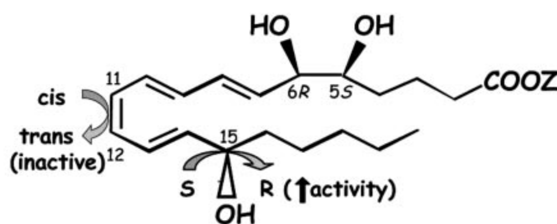
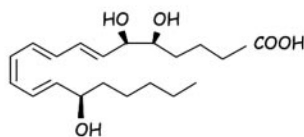
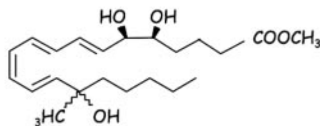


• LXA_4 (Z = H) and methyl ester (Z = CH_3)

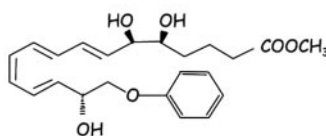
• 15-epi-LXA_4



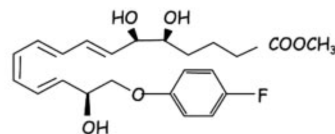
• 15(*R*/*S*)-methyl-LXA_4 [ATLa$_1$]



• 16-phenoxy-LXA_4



• 15-epi-16-(para-fluoro)-phenoxy-LXA_4 [ATLa$_2$]



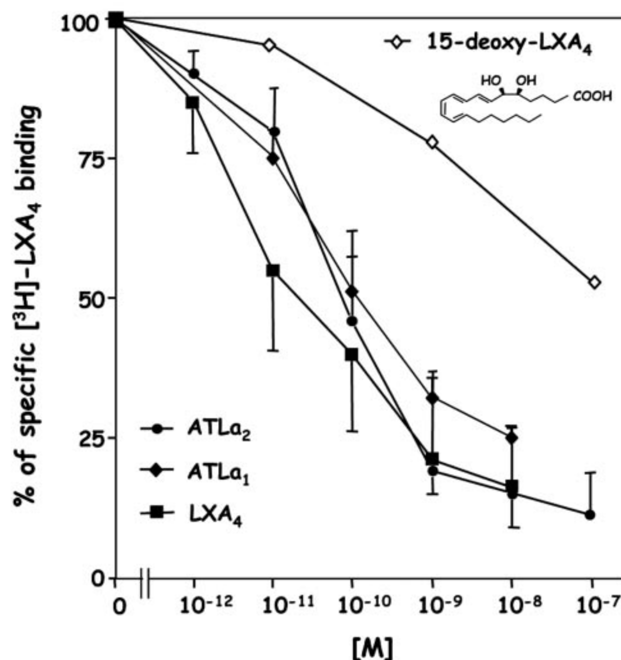


FIG. 2. LX-ALX interactions: structure-activity relationship of lipoxin and analogs. The LXA_4 interaction with ALX is highly stereospecific; that is, the 5*S*,6*R*-orientation of the two hydroxyl groups as well as 11-*cis* double bond conformation are essential for bioactions. 15-Epi-LXA_4 (ATL) carries a C-15 alcohol at the *R* configuration, opposite to the *S* configuration in native LXA_4, and was shown to have higher potency than native LXA_4 in certain bioassays. In 15(*R*/*S*)-methyl-LXA_4 (ATLa1), hydrogen at C-15 was replaced by a methyl group as a racemate at C-15. 16-Phenoxy-LXA_4 has a phenoxyl group at C-16 replacing the ω-end of the molecule to protect from dehydrogenation and ω-oxidation. In addition, fluoride was added to the *para* position of the phenoxy ring to hinder its degradation [ATLa2, 15-epi-16-(*para*-fluoro)-phenoxy-LXA_4] (left side). These compounds, which are more resistant to rapid dehydrogenation by 15-hydroxyprostaglandin dehydrogenase than native LXA_4, compete for $[^3H]LXA_4$ specific binding on PMNs and recombinant ALX (right side; see Chiang et al., 2000 for further details) and are potent inhibitors of PMN functions in vitro and in vivo.

III. Molecular Characterization of ALX, the Specific Receptor for Lipoxin A_4

A. Identification and Molecular Cloning of Human ALX: Cell Type-Specific Expression

To address the sites of action of LXA_4, radiolabeled $[11,12\text{-}^3H]LXA_4$ was synthesized and characterized (Brezinski and Serhan, 1991). By using this radioligand, the specific LXA_4 binding sites were first characterized on human PMNs (Fiore et al., 1992) and demonstrated to be responsible for the specific LXA_4-evoked actions on these cells. Intact PMNs exhibit specific $[11,12\text{-}^3H]LXA_4$ binding (K_d = 0.7 nM) that is inducible in promyelocytic lineage (HL-60) cells when exposed to differentiating agents (e.g., retinoic acid, dimethyl sulfoxide, and phorbol 12-myristate 13-acetate) and confers LXA_4-stimulated phospholipase activation (Fiore et al., 1993). In a parallel approach, receptors known to be induced within this time course (5 days) were screened for $[^3H]LXA_4$ binding and GTPase function. One of the orphan GPCRs cloned earlier from myeloid lineages (also known as FPRL1 and FPR2) was found to display specific $[^3H]LXA_4$ binding. LXA_4 exhibits high-affinity binding with this orphan GPCR and displays selectivity compared with other eicosanoids, including LXB_4, LTB_4, LTD_4, and PGE_2. The plasmid DNA of this orphan receptor denoted at the time as pINF114 was transfected into Chinese hamster ovary (CHO) cells and gave a K_d value of ~1.7 nM for $[^3H]LXA_4$ that was determined by Scatchard plot analysis (Fiore et al., 1994). This K_d value for recombinant pINF114/FPRL1 is comparable with values obtained with the endogenous LXA_4-specific binding sites present on peripheral blood human PMNs (K_d = 0.7 and 0.8 nM for isolated plasma membrane- as well as granule membrane-enriched fractions). In addition, using these pINF114-transfected CHO cells, LXA_4 stimulates both GTPase and the release of esterified AA in a pertussis toxin (PTX)-sensitive manner.

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Human ALX was subsequently identified and cloned in several types of leukocytes, including monocytes (Maddox et al., 1997) and T cells (Ariel et al., 2003), as well as resident cells such as macrophages, synovial fibroblasts (Sodin-Semrl et al., 2000), and intestinal epithelial cells (Gronert et al., 1998). Northern blot analysis demonstrates that the human ALX mRNA is ~2.1 kb (Fiore et al., 1994) and chromosome mapping reveals that the gene encoding human ALX is located on chromosome 19q (Bao et al., 1992). Together, these reconstituted functional responses that correlated with specific LXA_4 binding permit the assignment of this functional ligand-receptor pair (LXA_4-ALX) (Fiore et al., 1994). Hence, the above outlined systematic approach of screening orphan receptors using both function and specific binding of receptors that are induced during myeloid cell differentiation is the first identification of the high-affinity ligand for this receptor. A similar approach was also used later to identify the LTB_4 receptor (BLT1) (Yokomizo et al., 1997).

After FRPL1 was initially cloned as an orphan receptor, its cDNA was found to display high DNA sequence homology (~70%) to the FPR (Boulay et al., 1990). Hence, on the basis of DNA sequence homology alone, this receptor was considered to be like FPR and thus named FPRL1 (FPR-like 1) (Murphy et al., 1992), FPRH1 (Bao et al., 1992), and also FPR2 (Ye et al., 1992) or RFP (receptor related to FPR) (Perez et al., 1992). This receptor was also cloned by Nomura et al. (1993) from the human cDNA library and remained as an orphan receptor denoted by these investigators as HM63 (Table 1). The first agonist identified for FPRL1/ALX was fMLF (Ye et al., 1992). In this report, FPRL1 expressed in transfected cells mediated formyl peptide-stimulated calcium mobilization at micromolar concentrations of ligand. Thus, this GPCR does not effectively respond to fMLF in vitro or in vivo unless cells are exposed to higher pharmacological doses (i.e., >1–10 mM), suggesting that the formyl peptide fMLF is not a physiologically relevant ligand. Thus, the name FPRL1 does not reflect the true nature of this receptor.

B. Murine Homologs of ALX

The mouse ALX cDNA was cloned from a spleen cDNA library (Takano et al., 1997). Expressed in CHO cells, this mouse clone displays specific $[^3H]LXA_4$ binding (K_d = 1.5 nM determined by Scatchard plot analysis) and LXA_4-initiated GTPase activity (Takano et al., 1997) (Table 2). Northern blot analysis demonstrated that the mouse ALX mRNA is ~1.4 kb (Takano et al., 1997). An ortholog of ALX was isolated more recently from rat leukocyte and proved to be functional as demonstrated by radioligand binding and LXA_4-dependent inhibition of TNF-α-mediated NF-κB activity (Chiang et al., 2003). One should remember that at least nine distinct mouse genes in the FPR family have been cloned and are designated *Fpr1*, *Lxa4r/Fprl1,* and *Fpr-rs1* to *Fpr-rs7*. *Fpr1* is the ortholog of human FPR (Gao and Murphy, 1993). *Lxa4r/Fprl1* encodes a functional receptor for LXA_4 (Takano et al., 1997). *Fpr-rs1* and *Fpr-rs2* are most similar to mouse ALX (*Lxa4r/Fprl1*), sharing 97 and 83% identity, respectively, to mouse ALX in the deduced amino acid sequences (Gao et al., 1998). Importantly, *Fpr-rs1* differs from FPRL1 by the deletion of four amino acids at the cytoplasmic end of transmembrane domain 4, which is expected to alter the positioning of the domain in membrane and might have an impact on receptor function. Whether *Fpr-rs1*could bind and signal with LXA_4 remains of interest. *Fpr-rs2* was proposed to be a low-affinity receptor for fMLF (also referred to as FPR2) (Hartt et al., 1999). Three other genes (*Fpr-rs3, Fpr-rs4,* and *Fpr-rs5*) lack human counterparts and are currently considered orphan receptors. Recently, by screening a mouse genomic library, Wang and Ye (2002) reported two additional genes in this family, *Fpr-rs6* and *Fpr-rs7*. These two genes are 53 to 74% identical in amino acid sequences to other genes in the mouse FPR family; however, the ligands for these two putative receptors are currently unknown. Along these lines, by screening of a mouse macrophage cDNA library, Vaughn et al., (2002) reported several FPR-related clones. Among them, one clone (8C10), identical to *Fpr-rs2*, shares the highest identity with murine ALX (*Lxa4r/Fprl1*) at both nucle-

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TABLE 2
Comparison among human, mouse, and rat orthologs of ALX

The murine homologs Fpr-rs3 to Fpr-rs7 lack human counterparts, and their expression and function are not currently known. Arrows indicate either ↑ increased or ↓ decreased activity.

Species	Human			Mouse			Rat
Receptor		ALX		*Lxa4r/fprl1*	*Fpr-rs1*	*Fpr-rs2/8C10*	ALX
Cell type	PMN	Monocyte	Recombinant systems		Recombinant systems		Recombinant systems
Specific binding	LXA_4		LXA_4	LXA_4	N.D.	FMLP (low affinity)	LXA_4
LXA_4-signaling	↑ AA release ↑ PLD	↑ $[Ca^{2+}]$	↓ NF-κB	↑ GTPase ↑ IP3	N.D.	↑ IP3	↓ NF-κB
Bioaction	Anti-inflammation and proresolution						

N.D., not determined.

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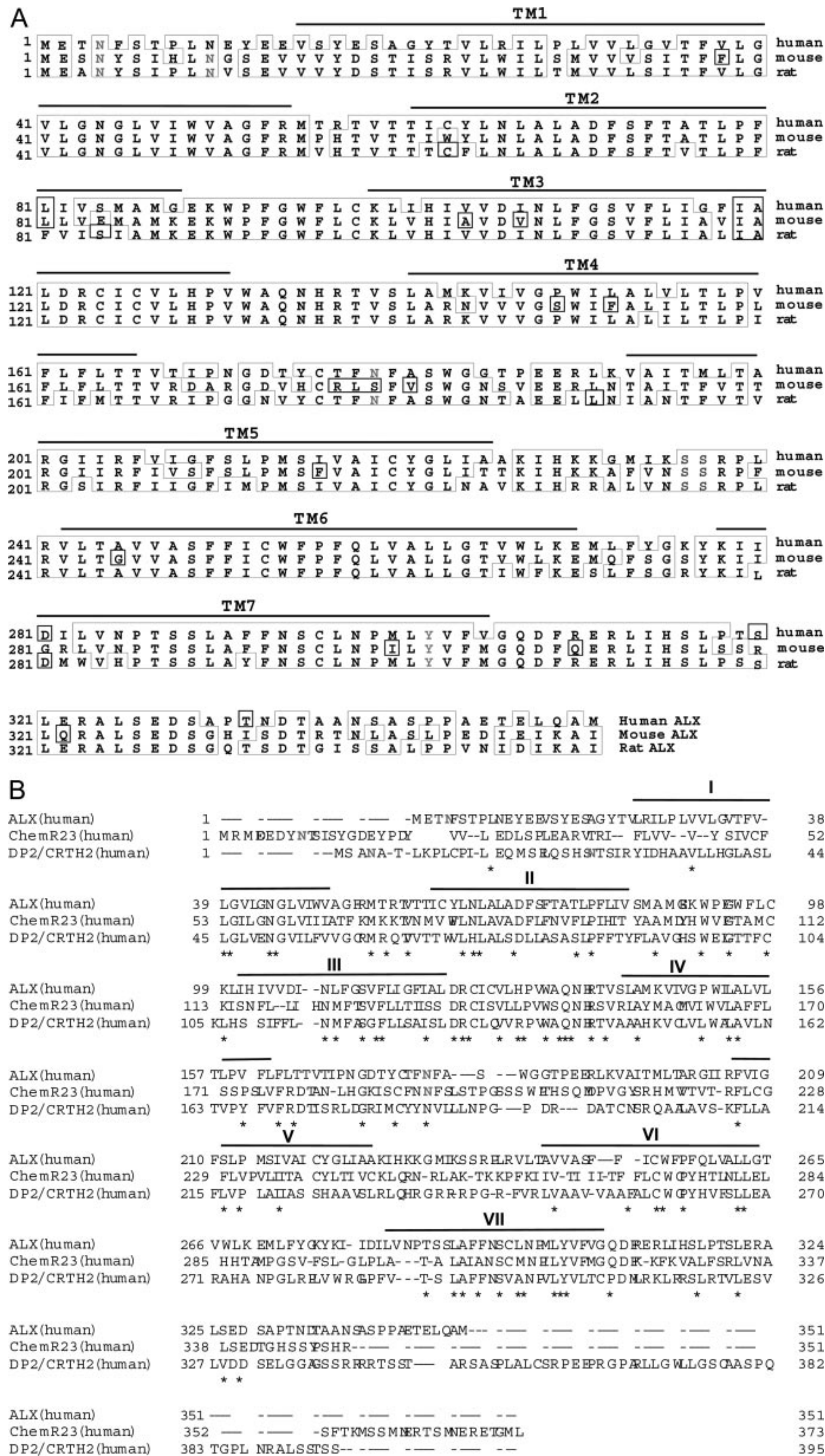

FIG. 3. Sequence alignments and phylogenetic tree of ALX and related GPCRs. A, alignment of deduced amino acid sequences of ALX: human, mouse, and rat. The identical amino acid residues in three species are boxed. The proximate positions of the putative TMs are lined and potential *N*-glycosylation and phosphorylation sites indicated. B, alignment of the deduced amino acid sequences of human ALX, DP_2/CRTH2, and ChemR23. Asterisks indicate conserved amino acids. Putative transmembrane domains are lined and labeled as I to VII.

otide and protein levels (89 and 83%, respectively) and functionally responds to LXA_4 as a ligand. One might note that in human the FPR, FPRL1, and FPRL2 gene cluster spans approximately 80 kb, whereas in mouse it covers almost 2 megabases with numerous partial gene duplications. Given the more complex genetics in mouse

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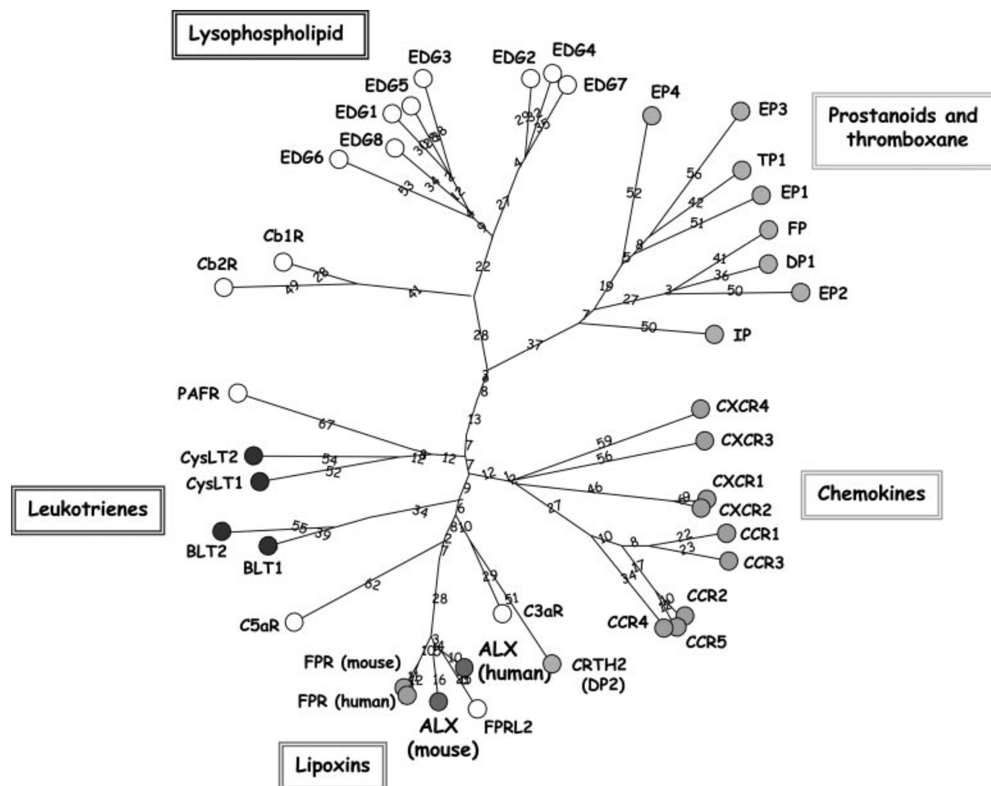

FIG. 4. ALX receptor cluster. The phylogenetic tree is constructed with deduced amino acid sequences of human ALX and related GPCRs using the All All Program at the Computational Biochemistry Server at Eidgenössiche Technische Hochschule Zürich (http://mendel.ethz.ch:8080/) TP, thromboxane A_2 receptor; DP1 and DP2, prostaglandin D_2 receptors; EP_1, EP_2, EP_3, and EP_4, subtypes of prostaglandin E_2 receptors; FP, prostaglandin F_2 receptor; IP, prostacyclin receptor, Cb1R and Cb2R, cannabinoid receptors.

relative to human, apparently the functional human ALX/FPRL1 has been assigned to separate mouse receptors: 1) *Lxa4r/Fprl1* (Takano et al., 1997; Vaughn et al., 2002) and 2) *Fpr-rs2* (also known as the 8C10 clone) (Vaughn et al., 2002) (Table 2).

C. Structure-Function Relationship of ALX: Receptor Chimeras for ALX-BLT

The deduced amino acid sequence for ALX places it within the GPCR superfamily characterized by seven putative TMs with an N terminus on the extracellular side of the membrane and a C terminus on the intracellular side (Baldwin, 1993). Human (Fiore et al., 1994), mouse (Takano et al., 1997), and rat (Chiang et al., 2003) ALX cDNA all contain an open reading frame of 1053 nucleotides, which encode a protein of 351 amino acids. The overall homology between human, mouse, and rat ALXs is 74% in nucleotide and 65% in deduced amino acid sequences (Fig. 3A). An especially high homology is evident in their second intracellular loop (100%) and between their corresponding sixth TM (97%) followed by the second, third, and seventh TMs as well as the first extracellular loop (87–89%). These conserved sequences among the human and murine receptors strongly suggest the essential roles for these regions in ligand recognition and functional G protein coupling. As a class, human, mouse, and rat ALX is only distantly related to the prostanoid receptors (Narumiya et al., 1999) and belongs to the cluster of chemoattractant receptors exemplified by receptors for fMLF, C5a, and platelet-activating factor and now also include LTB_4 receptors (BLT_1 and BLT_2), DP_2 (CRTH2), and ChemR23 (Fig. 3B and 4).

To evaluate the contributions of the major domains of ALX in interacting with either lipid or peptide ligands, chimeric receptors were constructed from receptors with opposing functions, namely ALX and BLT_1. These chimeras reveal that the seventh TM and adjacent regions of ALX are essential for LXA_4 recognition, and additional regions of ALX (e.g., extracellular loops) are required for high-affinity binding of the peptide ligands (i.e., MMK-1 and MHC binding peptide). These findings are the first to indicate that a single GPCR can recognize and function with specific chemotactic peptides as well as lipid-derived ligands, LXA_4, but clearly act with different affinities and/or distinct interaction sites within the receptor, ALX (Chiang et al., 2000) (see section IV for details on peptide ligands of ALX). Conserved *N*-glycosylation sites are present on Asn-4 and Asn-179 of human ALX. Bacterial and viral infections interfere with normal *N*-glycosylation of the host cells (Olofsson et al., 1980; Kim and Cunningham, 1993; Villanueva et al., 1994), and this characteristic of the ALX receptor for ligand class recognition was of interest to assess. In this regard, deglycosylation of ALX does not dramatically

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alter LXA_4 recognition, but significantly lowers the affinity for ALX peptide ligands (i.e., MMK-1 and MHC binding peptide) (Chiang et al., 2000). Thus, *N*-glycosylation is important for ligand specificity of this receptor and may play a role in switching receptor functions at local host defense sites.

Along these lines, several conserved motifs and amino acid residues important for post-translational modification are found in ALX. For example, Ser-236, Ser-237, and Tyr-302 are essential for human ALX phosphorylation and signaling. Site-directed mutagenesis of these residues in ALX was carried out, and expression of the wild-type and mutated ALX in CHO and HL-60 cells was used to examine the ligand-receptor interactions and signal transduction events. Results indicate that mutation of ALX at either serine (Ser-236 and -237) or tyrosine (Tyr-302) phosphorylation sites displays sustained activation of phospholipase A_2 and D in contrast to the transient activation obtained with wild-type ALX (Kang et al., 2000). Together, these structure-function studies of ALX (i.e., receptor chimera, glycosylation, and phosphorylation) not only identify the key domains/residues for ligand interaction and signaling, but also further substantiate the fact that LXA_4 is the highly stereospecific ligand for the receptor ALX.

IV. Flexibility of G Protein-Coupled Ligand-Receptor Interaction: Lipid- versus Peptide-Derived Ligands

A. Sequence Homologies and Ligand-Receptor Diversity

Results obtained from different experimental systems collectively indicate that specific small peptides as well as bioactive lipid mediators can both function as ligands for the same receptor. These interactions, however, occur with different affinities and may be at distinct sites within the receptor and evoke separate intracellular signaling that depends on the cell type and system. The intracellular protein interactions after ligand-receptor binding are different for peptide versus lipid ligands of ALX. In this context, there may be a similar scenario for two other related GPCRs: 1) GPR44 (G protein-coupled receptor 44, AF118265), also known as DP_2/CRTH2, and 2) CMKLR1 (chemokine-like receptor 1, U79526), also known as ChemR23. DP_2/CRTH2 was originally cloned as an orphan GPCR selectively expressed in Th2 cells, eosinophils, and basophils (Nagata et al., 1999). Based on the DNA sequence homology in comparison with members of the FPR subfamily (Fig. 3B and 4), this receptor was proposed to be a putative chemoattractant receptor and thus referred to as chemoattractant receptor-homologous molecule and was expressed on Th2 cells (CRTH2). Later, the lipid-derived mediator PGD_2 was found to be the specific, high-affinity ligand for CRTH2 (Hirai et al., 2001). PGD_2 is the major eicosanoid produced by activated mast cells and thereby has long been implicated in allergic diseases. CRTH2, but not the classic PGD_2 receptor (DP1), induces migration of Th2 cells, eosinophils, and basophils in response to PGD_2. Thus, CRTH2 is also termed DP_2 as the second PGD_2 receptor.

Along similar lines, a specific receptor for the ω-3 eicosapentaenoic acid-derived resolvin E1 (RvE1) was recently identified as the orphan receptor ChemR23 (Arita et al., 2005), which also belongs to this cluster of GPCRs. RvE1 and LXA_4 each have different structures and are formed via different biosynthetic pathways and precursors (eicosapentaenoic acid versus AA). Yet these two local mediators seem to share similar beneficial properties that lead to dampening of inflammation in vivo. The selective ligand, RvE1, which activates the recombinant ChemR23 receptor, inhibits NF-κB in human embryonic kidney (HEK) 293 cells. Of interest, ChemR23 shares an overall 36.4% identity with ALX in the deduced amino acid sequences with a highly conserved domain within the second intracellular loop with 75% identity, followed by the seventh TM with 69.5% identity (Fig. 3B). These highly conserved regions within structurally related GPCRs (i.e., ALX and ChemR23) might contribute to their anti-inflammatory and proresolving properties. Recently both RvE1 and a new peptide ligand generated from chemerin were demonstrated to serve as endogenous ligands for ChemR23, but transmit ligand-dependent signals dictating different functional responses in vivo (Arita et al., 2005). Thus, ALX and ChemR23 not only share overall structure homology and highly conserved regions but also display functional similarities in their ligand recognition properties (i.e., interacting with both lipid- and peptide-derived ligands). Taken together, the "dual ligand" properties might be conserved in these structurally and functional related GPCRs. In addition, the findings that specific endogenous lipid mediators and certain peptides interact with the same receptor may reflect at the genomic level the economy of using one receptor structure for multiple recognitions and functions in the immune system.

B. ALX and Structurally Unrelated Peptide Ligands in Vitro

ALX is stereoselective for the eicosanoid-based ligands. In comparison, many peptides/proteins can interact with ALX in vitro to activate calcium mobilization (Table 3; Fig. 5). These include bacteria- and host-derived peptides, HIV-1 envelope proteins/peptides, and neurotoxic as well as synthetic peptides. Some of the naturally produced peptides interact with ALX in the nanomolar to subnanomolar range. For example, the nonformylated MHC binding peptide (a potent necrotactic peptide derived from NADH dehydrogenase subunit 1 from mitochondria) directly binds to human ALX with high affinity (competing with $[^3H]LXA_4$ with an $IC_{50} < 10^{-9}$ M), and evokes PMN chemotaxis that is stopped by LXA_4 (Chiang et al., 2000). This peptide also stimulates macrophage phagocytosis of PMNs (Mitchell et al.,

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TABLE 3
Lipid and peptide ligands for FPR, ALX/FPRL1, and FPRL2

Ligand	FPR		FPRL1		FPRL2	
	K_d	EC_{50}	K_d	EC_{50}	K_d	EC_{50}
Lipid-derived agonists						
LXA$_4$ and ATL	5 μM		1.7 nM			
Bacteria-derived peptides						
fMLF	1 nM		>1 μM		>1 μM	
Hp(2–20)				300 nM		
Host-derived peptides						
uPAR fragment			83 nM			
Annexin 1			900 nM			
MHC binding peptide			1 nM			
SAA			45 nM			
LL-37				5 μM		
CKβ8-1		>1 μM		1 nM		1 μM
F2L		>1 μM		>1 μM		10 nM
HIV-1 envelope peptides						
T20/DP176						
T21/DP107				500 nM		
N36				10 μM		
F peptide				10 μM		
V3 peptide				1.5 μM		
Synthetic peptides						
MMK-1		10 μM		2 nM		
WKYMVm		1 nM		75 pM		3 nM
Neuropeptides						
PrP106–126				10 μM		
Aβ42				1.5 μM		
Humanin				3.5 nM		

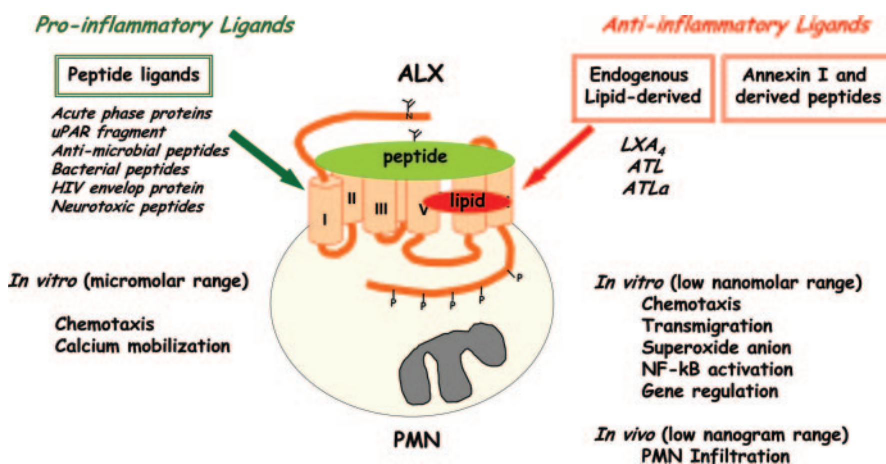

FIG. 5. Bioactions of lipid versus peptide ligands for ALX.

2002). A mitochondria peptide fragment such as MYFINILTL derived from NADH dehydrogenase subunit 1 (ND1) directly stimulates PMN chemotaxis. This peptide binds to the MHC class Ib molecule H2-M3 in formylated as well as nonformylated forms and thus is denoted as MHC binding peptide. The mitochondria-derived peptides, including ND1 peptides, are held to be liberated from mitochondria and may play a role in accumulation of phagocytic cells during tissue and cell lysis that can accompany bacterial infection and/or ischemia-reperfusion injury. In this context, a similar observation that *N*-formylated peptides corresponding to endogenous mitochondrial protein sequences are able at low nanomolar concentrations to activate the receptor ALX was recently reported by Rabiet et al. (2005). In addition, the naturally cleaved form [i.e., D2D3(88-274)] of urokinase-type plasminogen activator directly binds to ALX and is a unique endogenous chemotactic agonist for ALX, providing the first direct link between the fibrinolytic machinery and the inflammatory response (Resnati et al., 2002).

Additional peptides in the micromolar range can also interact with ALX in some in vitro model systems; these include HIV envelope peptides (0.5–10 mM) (e.g., T21, N36, V3, and F peptides), antimicrobial peptides (e.g., LL37 and temporin A), truncated chemotactic peptides (e.g., CKβ8-1), and bacterial-derived peptides (e.g., Hp2-20 from *Helicobacter pylori*) as well as host-derived peptides (e.g., SAA, PrP106-126, and amyloid β peptide Aβ42) (reviewed in Le et al., 2002; Fu et al., 2006). These

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peptides evoke calcium mobilization and chemotaxis in vitro with recombinant ALX; however, the functional role(s) of these peptides in human biology remains to be determined. In addition, note that LXA_4 stimulates nonphlogistic chemotaxis of monocytes and calcium mobilization that is not required for monocyte functions (Maddox et al., 1997), suggesting that calcium mobilization is an epiphenomenon in these cells. In addition, these peptides/proteins do not share apparent homology in terms of the primary amino acid sequences. Thus, the common structural requirements (e.g., tertiary conformation) for peptide ligands to interact with ALX remain of interest. They also raise the question of whether calcium mobilization alone is an appropriate second messenger in vitro to gain information on the physiologic ligands for ALX in vivo.

C. Emergence of Endogenous Anti-Inflammatory Peptides

A recent report demonstrated that glucocorticoid-induced annexin I (ANXAI) and its derived peptides (e.g., Ac2-26) generated in vivo act at the ALX to halt PMN diapedesis (Perretti et al., 2002; Hayhoe et al., 2006) (Fig. 5). ANXAI and ANXAI-derived peptides interact directly with recombinant human ALX, as demonstrated by radioligand binding and function as well as by immunoprecipitation of PMN receptors. In addition, the combination of both ATL (see section VI. for details) and Ac2-26 limits PMN infiltration and reduces production of inflammatory mediators in murine dorsal air pouches. Along these lines, both ALX agonists, namely, ATL and Ac2-26, induce detachment of adherent leukocytes in the mesenteric microcirculation (Gavins et al., 2003) and promote phagocytosis of apoptotic PMNs by human macrophages (Maderna et al., 2005). Ac2-26 also attenuates acute myocardial injury. This action does not involve FPR, as the cardioprotection by Ac2-26 was intact in FPR-null mice. In comparison, an LXA_4 analog also exerted cardioprotection in both wild-type and FPR-null mice, suggesting a pivotal role for ALX in acute cardioprotection (Gavins et al., 2005). Thus, by convergence at the same anti-inflammatory receptor, these two structurally distinct endogenous systems, namely, lipid-derived (e.g., ATL) and protein-derived (e.g., ANXAI) mediators, limit PMNs and promote resolution in vivo. These systems probably represent functional redundancies in endogenous anti-inflammation circuits that unveil presently unappreciated mechanisms operative in governing PMN responses in host defense and open new avenues for therapeutic approaches such as combination therapies.

D. Similarity of Receptor and Ligand Structures in the FPR Family

1. Receptors. The three FPR-related receptors (i.e., FPR, FPRL1, and FPRL2) share significant sequence homology among themselves but have different pharmacological properties, especially when ligand binding is considered. It is also worth noting that the sequence for ALX/FPRL1 is 70% identical overall to its closest homolog, the formyl peptide receptor FPR, and that the intracellular domain sequences for the two receptors are >90% identical. They have both been shown to activate pertussis toxin-sensitive G proteins, and this has been shown for both fMLF and LXA_4 in the case of ALX/FPRL1.

2. Ligands. Peptide ligands for the FPR family are different from chemokine receptors because the peptide ligands, unlike chemokines, have neither fixed cysteine residues nor a fixed size of 8 to 10 kDa. Instead, these peptide ligands share very little sequence homology among each another, other than the *N*-formyl group for most of the FPR ligands, and within this context there are similarities between peptide fMLF itself and the LXA_4 structure. Based on three-dimensional modeling, the C17 to C20 of LXA_4 is similar to the side chain of the leucine residue in fMLF (Mills et al., 1999). In addition, based on site-directed mutagenesis, photocross-linking, and three-dimensional modeling, fMLF can be placed within the binding pocket between the seven transmembrane helices (Miettinen et al., 1997), a characteristic similar to the behavior of the eicosanoid receptors and distinct from that of the chemokine receptors. Together, these findings might explain why ALX/FPRL1 can bind fMLF with low affinity (>1 μM).

V. ALX: Historical Perspective and Connection to the FPR

A. Discovery of N-Formylated Peptides and the FPR: Why Not Keep the Name FPRL1 for This Receptor?

In the early 1950s, viable bacteria in infected tissues were observed to attract PMNs, presumably by releasing chemoattractant factors (Harris, 1954). Later Schiffmann et al. (1975) found that supernatants of *Escherichia coli* cultures contain *N*-formylated di- and tripeptides that are chemoattractants for phagocytes. From the systematic analysis of synthetic peptides, the surrogate tripeptide fMLF was identified as the shortest sequence module to activate phagocyte functions (Showell et al., 1976; Freer et al., 1980, 1982). Besides bacteria, the mitochondrial protein synthetic apparatus in mammalian cells also initiates protein synthesis with *N*-formylmethionine that is retained at the amino terminus of several purified mitochondrial proteins (Carp, 1982). In this context, *N*-formylated peptides could be released from degenerating mitochondria at the sites of tissue damage and play a role in the accumulation of inflammatory cells. The genome of human mitochondria is unique in that exceptions to the universal genetic code occur in the mitochondria. Many of the changes affect codons involved in either initiating or terminating protein synthesis. In addition, the mitochondria genome

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provides some striking connections between prokaryotic and eukaryotic worlds. Because there are many general similarities between mitochondria and bacteria, mitochondria may have been derived from the microbial world and hence encode *N*-formylated proteins that are not found in appreciable amounts elsewhere in human cells. Indeed, Carp (1982) demonstrated that the isolated disrupted human mitochondria stimulate PMN chemotaxis in vitro, possibly via the mitochondrially derived *N*-formylated proteins/peptides. This earlier finding may be important in apoptosis and clearing of cellular debris by phagocytes—a process called necrotaxis. Exposure of the mitochondrial proteins in vivo may lead to leukocyte accumulation followed by accelerated phagocytosis. In this context, it is noteworthy that LXA_4 is a potent chemoattractant for monocyte recruitment in vitro (Maddox et al., 1997) and in vivo (Hachicha et al., 1999). In addition, LXA_4 stimulates macrophage uptake of apoptotic PMNs (Godson et al., 2000).

The *N*-formylated peptide fMLF binds with high affinity to a specific GPCR, which was identified (Schiffmann et al., 1975) and cloned in 1990 (Boulay et al., 1990). This cloned receptor shared properties of the receptors characterized via biochemical approaches (Jesaitis et al., 1982) and was then termed formyl peptide receptor (FPR). Two additional human genes have also been isolated by low-stringency cross-hybridization with human FPR, namely FPRL1 and FPRL2. Because fMLF interacts with FPR on phagocytic cells, the assumption was made that a phagocytic or chemotactic response in host defense might also be associated with the sequence-related receptors such as FPRL1 (Boulay et al., 1990; Bao et al., 1992; Ye et al., 1992; Nomura et al., 1993). Among these, Ye et al. (1992) first identified fMLF as a low-affinity ligand for FPRL1. Only relatively high concentrations (i.e., >1–10 μM) can bind and stimulate calcium mobilization with FPRL1, results that suggest that the formyl peptide fMLF is not a physiologically relevant ligand for FPRL1. The putative endogenous FPR ligands remain to be identified, that is, those endogenous peptides encoded by the human genome that are possibly present in the blood and/or bone marrow to regulate leukocyte trafficking. Thus, the name FPRL1 does not reflect the in vivo properties found for this receptor. For some of the receptors, ligands could very well be exogenous and have low affinities, as is the case with ligands for Toll-like receptors and odorant receptors. However, if an endogenous and high-affinity ligand is discovered for such a receptor, the receptor should be renamed after this ligand. In this case, we review here in the following sections the compelling evidence for the ligand-receptor interaction in vitro and in vivo that substantiates this GPCR (FPRL1/ALX) being named ALX.

B. What Are the Lines of Evidence and Criteria That Justify This G Protein-Coupled Receptor Being Named ALX?

1. Direct Binding and Signaling with Lipoxin A_4: Recombinant Systems. The orphan receptor FPRL1 was originally named and categorized into the FPR family based only on its high sequence homology to FPR. However, FPRL1 binds fMLF with very low affinity (K_d = 5 μM) (Fiore and Serhan, 1995). In sharp contrast, recombinant FPRL1 (1) specifically binds LXA_4 with high affinity (K_d = 1.7 nM) [Fiore et al., 1994 (human); Takano et al., 1997 (human and mouse); Chiang et al., 2000 (human), 2003 (rat); Perretti et al., 2002 (human); Gronert et al., 2001 (human)] and (2) transmits signal with the ligand LXA_4, including stimulation of both GTPase and the release of esterified AA (Fiore et al., 1994; Takano et al., 1997), evoking chemotaxis (Chiang et al., 2000; Perretti et al., 2002) and inhibition of TNF-α-mediated NF-κB activity (Chiang et al., 2003). These results indicate that this cDNA encodes a functional receptor for LXA_4 that activates robust and specific intracellular signals with this ligand and thus is denoted ALX (see section III. for detailed molecular characterization of ALX).

2. Lipoxin A_4 Is the Endogenous Agonist with High Potency. The lipid-derived ligand LXA_4 is 1) generated endogenously in animal models and in humans (see sections II. and VI.) and 2) displays potent protective actions in the low nanomolar range via ALX in a variety of animal models of diseases (see section VII.). In contrast, certain peptides/proteins can also interact with ALX (Table 3; Fig. 5). Most of them are either fragments of endogenous peptides/proteins such as fMLF or synthetic peptides and activate ALX in the micromolar range only in specific in vitro model systems (see section IV.). The functional role(s) of these peptides in human biology remains questionable since further work is required to clarify when and where they are produced in vivo.

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TABLE 4
Major points that justify the nomenclature for ALX in preference to FPRL1

ALX	FPRL1
Direct [^{3}H]LXA_4 binding and LXA_4-dependent signaling events	Based on only DNA sequence homology; this receptor does not effectively bind and respond to fMLF
The ligand LXA_4 is generated in vivo	Most of the peptide ligands are either protein fragments or surrogate or synthetic peptides; their presence in vivo is surmised
LXA_4 evokes specific bioactions in vitro and in vivo in the low-to-subnanomolar range	Most of the peptide ligands evoke signals in vitro in the micromolar range
SAR—highly stereoselective in that the essential functional groups of LXA_4 and ATL for receptor activation are also pharmacophores for their in vivo anti-inflammatory actions	No sequence similarities between peptide ligands; the structure requirement for receptor interaction is not available

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3. Structure-Activity Relationship in Vitro and in Vivo. The action of LXA_4 is highly stereoselective in terms of the alcohol chirality, double bond geometry, and requirement for overall conformation. The key structure requirements to activate ALX include the presence of a 15-hydroxyl group, a tetraene structure, and an 11,12-*cis* as well as a 13,14-double bond within the tetraene. These are also the pharmacophores for the anti-inflammatory actions of LXA_4. Therefore, we propose to keep the official nomenclature for this LXA_4-activated receptor as ALX (Fiore et al., 1994; reviewed in Brink et al., 2003), since the endogenous lipid-derived mediator LXA_4 is the most potent agonist. Table 4 outlines the major points that justify the nomenclature for ALX in preference to FPRL1.

VI. "Aspirin-Triggered" Lipoxin-Generating Systems

A. Transcellular Biosynthesis via Acetylated Cyclooxygenase-2

Whereas many of the therapeutic benefits of aspirin are attributable to the inhibition of COX and the biosynthesis of PG and thromboxane, aspirin is also known to initiate the generation of endogenous anti-inflammatory lipid mediators, namely, aspirin-triggered lipoxins. This action is documented in COX-2-bearing cells, such as vascular endothelial cells or epithelial cells and their coactivation with PMNs (Fig. 1). Briefly, inflammatory stimuli (e.g., TNF-α or lipopolysaccharides) up-regulate COX-2. When aspirin is administered, COX-2 is irreversibly acetylated but remains active and changes the products of the enzyme from intermediates for PG and TX to precursors for ATL, namely 15*R*-HETE. This precursor carries a carbon 15 alcohol in the *R* configuration and is rapidly converted by 5-LO in activated PMN to 15-epimeric-LXA_4, or ATL, that carries the C-15 hydroxyl group in the *R* configuration rather than 15*S* native LXA_4 (Clària and Serhan, 1995). 15-Epi-LXB_4 also carries a 15*R* alcohol (Fig. 1) and shows activities similar to those of 15-epi-LXA_4 in some biologic systems. In many other settings, 15-epi-LXA_4 and 15-epi-LXB_4 each display distinct actions. 15-Epi-LXB_4, for example, is a more potent inhibitor of cell proliferation than LXA_4 or 15-epi-LXA_4 (Clària et al., 1996).

B. Formation of Aspirin-Triggered Lipoxin in Vivo

Using both a specific enzyme-linked immunosorbent assay method and the liquid chromatography-tandem mass spectrometry system, ATL has also been detected in vivo in an aspirin-dependent manner in murine peritonitis (Chiang et al., 1998) and dorsal air pouches (Perretti et al., 2002), as well as in rat kidney (Munger et al., 1999) and liver (Titos et al., 1999). Furthermore, aspirin rapidly up-regulates COX-2 expression in the stomach and causes a significant increase in gastric ATL production in rats (Fiorucci et al., 2002). Alterations in ATL levels are also documented in human subjects. For example, ATL formation is established in ASA-tolerant and ASA-intolerant asthmatics (Bonnans et al., 2002). Of specific interest, aspirin at clinically relevant doses (81, 325, and 650 mg daily) in healthy subjects, increases anti-inflammatory ATL levels as well as blocks thromboxane (Chiang et al., 2004). Thus, ATL formed in vivo provides a novel mechanism underlying the clinical benefits of aspirin, namely, the triggering of anti-inflammatory mediators from endogenous precursors that in turn dampen inflammation.

C. Structure-Activity Relationship of Aspirin-Triggered Lipoxin and Design of Stable Analogs

The ATL with the 15-hydroxyl group in the *R* configuration was shown to directly bind and signal with ALX, with more potent bioaction than the native 15*S*-LXA_4 in vitro and in vivo (Serhan et al., 1995; Takano et al., 1997; reviewed in Serhan, 1997). As is the case for other autacoids, both LXA_4 and ATL are rapidly generated in response to stimuli, act locally, and then are rapidly inactivated by metabolic enzymes. Thus, stable analogs were constructed with specific modifications of the native structures of LXA_4 and ATL. These compounds resist enzymatic metabolism, maintain the structural integrity, and exhibit potent enhancement in bioactivity. For example, 15(*R*/*S*)-methyl-LXA_4 is a racemic stable analog of both LXA_4 and ATL that is not rapidly metabolized to the 15-oxo-LXA_4 and hence enhances bioaction in vitro (Serhan et al., 1995) and in vivo (Takano et al., 1997). Additional analogs of LXA_4 and ATL were synthesized with a phenoxy group bonded to carbon 16 replacing the ω-end of the molecule to protect from both dehydrogenation and ω-oxidation in vivo. Fluoride was added to the *para*-position of the phenoxy ring to hinder its degradation (Fig. 2). These modifications not only prolong the half-life of the compounds in blood but also enhance their bioavailabilities as well as potency in vivo (Clish et al., 1999). These analogs also act via interaction with endogenous as well as recombinant ALX (Chiang et al., 2000). Thus, these metabolic stable analogs serve as useful tools to investigate the action of LXA_4 and ATL in vitro and in vivo (Tables 5 and 6), and offer leads for developing novel therapeutic interventions.

VII. Biological Significance of the Lipoxin-ALX System

A. Bioactions of Lipoxin A_4 and Aspirin-Triggered Lipoxin A_4 in Animal Models of Diseases

In dermal inflammation, LX stable analogs when applied topically to mouse ears stop both PMN infiltration

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TABLE 5
Actions of LXA_4 and ATL in cellular/tissue systems

Cell Type/Tissue	Action
Whole blood	Down-regulate CD11b/CD18, prevent shedding of L-selectin and reduce peroxynitrite generation on PMNs, monocytes, and lymphocytes (Filep et al., 1999; Jozsef et al., 2002)
Neutrophils	Inhibit chemotaxis, adherence and transmigration (Serhan et al., 1995)
	Inhibit PMN-epithelial and PMN-endothelial cell interactions (Papayianni et al., 1996)
	Block superoxide anion generation (Levy et al., 1999)
	Reduce CD11b/CD18 expression and inositol triphosphate formation (Fiore and Serhan, 1995)
	Inhibit peroxynitrite generation (Jozsef et al., 2002)
	Attenuate AP-1 and NF-κB accumulation and inhibit IL-8 gene expression (Jozsef et al., 2002)
Monocytes	Stimulate chemotaxis and adhesion to laminin without increase in cytotoxicity (Maddox and Serhan, 1996; Maddox et al., 1997)
	Inhibit peroxynitrite generation (Jozsef et al., 2002)
	Inhibit IL-8 release by cells obtained from asthma patients (Bonnans et al., 2002)
Macrophages	Stimulate nonphlogistic phagocytosis of apoptotic PMNs (Godson et al., 2000; Mitchell et al., 2002)
Dendritic cells	Block IL-12 production (Aliberti et al., 2002a)
Eosinophils	Stop migration/chemotaxis in vivo and inhibit eotaxin and IL-5 generation (Lee et al., 1989; Bandeira-Melo et al., 2000a,b)
Activated T cells	Inhibit TNF-α secretion by blocking ERK activation (Ariel et al., 2003)
NK cells	Block cytotoxicity (Ramstedt et al., 1987)
Myeloid progenitors	Stimulate myeloid bone marrow-derived progenitors (Stenke et al., 1991)
Enterocytes	Inhibit TNF-α-induced IL-8 expression and release (Gewirtz et al., 2002)
	Inhibit *S. typhimurium*-induced IL-8 (Gewirtz et al., 1998)
Fibroblasts	Inhibit IL-1β-induced IL-6, IL-8, and MMP-3 production (Sodin-Semrl et al., 2000)
Endothelia (HUVECs)	Stimulate protein kinase C-dependent prostacyclin formation (Brezinski et al., 1989)
	Block P-selectin expression (Scalia et al., 1997)
Mesangial cells	Inhibit LTD_4-induced proliferation (McMahon et al., 2000)
Pulmonary artery	Induce relaxation and reverse precontraction by $PGF_{2\alpha}$ or endothelin-1 (Dahlén and Serhan, 1991)
Hepatocytes	Reduce peroxisome proliferator-activated receptor-α and CINC-1 expression levels (Planagumà et al., 2002)
Bronchi	Relaxation after precontraction by blocking peptido-leukotrienes in human airway (Christie et al., 1992)

TABLE 6
LXA_4 and ATL bioactions in animal models of disease

Disease Model	Bioaction(s)
Dermal inflammation	Inhibit neutrophil recruitment into ear skin; prevent vascular permeability
I/R injury	Attenuate hindlimb I/R-induced lung injury; detachment of adherent leukocytes in mesenteric I/R; reduce myocardial infarct size and area at risk in myocardial I/R (infarction)
Peritonitis	Inhibit neutrophil recruitment and vascular leakage; promote phagocytosis of neutrophil by macrophage
Colitis (IBD)	Attenuate proinflammatory gene expression and reduce severity of colitis; inhibit weight loss, inflammation, and immune dysfunction
Glomerulonephritis	Reduce leukocyte rolling and adherence; decrease neutrophil recruitment
Asthma	Inhibit airway hyper-responsiveness and pulmonary inflammation
Cystic fibrosis	Decrease neutrophilic inflammation, pulmonary bacterial burden, and disease severity
Angiogenesis	Reduce angiogenic phenotype: endothelial cell proliferation and migration
Periodontitis (oral inflammation and bone loss)[a]	Reduce microbe-initiated, neutrophil-mediated tissue damage, and bone destruction
Eye (wound healing)	Accelerate cornea re-epithelialization, limit sequelae of thermal injury (i.e., neovascularization, opacity), and promote host defense
BMT	Protect against BMT-induced graft vs. host disease

I/R, ischemia-reperfusion; IBD, inflammatory bowel disease; BMT, bone marrow transplant.
[a] All animal models were designed and carried out in either mice or rats, except for the periodontitis, which was carried out in rabbits. See text for original citations and further details.

and vascular permeability changes (Takano et al., 1997, 1998). In addition, the fluorinated analog of ATL [i.e., ATLa (*a*spirin-*t*riggered *L*XA_4 stable *a*nalog)] at levels as low as ~24 nmol/mouse, potently blocks TNF-α-induced leukocyte recruitment into the dorsal air-pouch (Clish et al., 1999). Inhibition by ATLa is evident via either local intra-air pouch delivery or systemic delivery by intravenous injection and proves to be more potent than local delivery of aspirin. Nearly, 1 mg of aspirin is needed to reach the level of reduction in PMN infiltration achieved by 10 μg of ATLa (Clish et al., 1999). In a peritonitis model in rats, ATLa also significantly reduces neutrophil infiltration (~43%) and protein extravasation (~42%) when given intravenously with two consecutive doses at ~60 μg/kg each injection (Chiang et al., 2003). More recently, ATLa showed an early protective role for host PMNs in allogeneic bone marrow transplant-induced graft-versus-host disease and delays death (Devchand et al., 2005). In addition, ATLa reduces splenic dendritic cell mobilization and the interleukin (IL)-12 response to *T. gondii*-derived pathogen-infected mice, demonstrating a further role for LXs in regulating proinflammatory responses during microbial infection (Aliberti et al., 2002a,b). Moreover, ATLa in a mouse model of the chronic airway inflammation and infection associated with cystic fibrosis suppresses neutrophilic

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inflammation, decreases pulmonary bacterial burden, and attenuates disease severity (Karp et al., 2004), indicating a potential role for LXs in this lethal autosomal disease. Recently, using a murine model of inflammatory pain, ATLa showed potent, dose-dependent analgesic action (J. M. Schwab et al., manuscript in preparation).

B. Lipoxin-ALX as a Protective Circuit in Vivo: Lessons from Genetically Engineered Animals

1. ALX and BLT Transgenic Mice. The protective actions of LXA_4 and ATL prove to be both ligand- and receptor-dependent. To address the potential for direct functional links between LXs and ALX in vivo, transgenic mice overexpressing human ALX (namely, the human receptor expressed in the mouse model) were prepared. These ALX transgenic mice give a profound anti-inflammatory phenotype, with markedly decreasing PMN infiltration into the peritoneum in zymosan A-initiated peritonitis, compared with their nontransgenic littermates (Table 7). They also display increased sensitivity to suboptimal doses of ATLa (Devchand et al., 2003). Along these lines, transgenic expression of human ALX in murine leukocytes leads to significant inhibition of pulmonary inflammation and eicosanoid-initiated eosinophil tissue infiltration, highlighting a unique counter-regulatory profile for the LXA_4-ALX system in airway responses (Levy et al., 2002). More recently, with an acute lung injury model, ALX transgenic mice also exhibit dramatic protection, suggesting potential therapeutic implications for this devastating clinical disorder (Fukunaga et al., 2005). Because LXA_4 and ATL selectively regulate leukocyte responses, they were tested in BLT1 transgenic mice that show dramatically increased PMN trafficking to lungs after high limb ischemia-reperfusion. Despite excessive PMN recruitment in BLT1 transgenic mice, intravenous injection of ATLa markedly diminishes reperfusion-initiated PMN trafficking to the lungs, revealing the protective role for LXA_4 and ATL in stress responses, which has applications in perioperative medicine (Chiang et al., 1999).

2. 15-Lipoxygenase Transgenic and Knockout Animals. The protective actions of LXA_4 are further substantiated using genetically engineered animals with altered expression and activity of 15-LO, an essential enzyme in one pathway for LXA_4 production (Fig. 6). The human 15-LO gene was transfected into rat kidneys in vivo in glomerulonephritis (acute or accelerated forms of antiglomerular basement membrane antibody-mediated). The glomerular functions (filtration and protein excretion) are preserved in 15-LO mRNA-transfected kidneys, but not in contralateral control kidneys or sham-transfected animals (Munger et al., 1999). These studies provide in vivo derived results supporting a direct anti-inflammatory role for 15-LO during immune-mediated tissue injury. In a recent study with transgenic rabbits overexpressing 15-LO type I in a macrophage-specific manner, LX production is significantly enhanced. Microbe-associated inflammation and leukocyte-mediated bone destruction were assessed in these rabbits by initiating acute periodontitis. 15-LO transgenic rabbits exhibit markedly reduced bone loss and local inflammation compared with their nontransgenic littermates (Serhan et al., 2003). These results indicate that overexpression of 15-LO type I and LXA_4 is associated with dampened PMN-mediated tissue degra-

TABLE 7
The protective roles of LXA_4-ALX circuits revealed by genetically engineered animals
See text for original citations and further details.

Knockouts or Transgenics	Disease Models	Phenotypes
Altered biosynthetic pathways		
15-LO transgenic rabbits	Periodontitis	Enhanced LXA_4 production
		Reduced bone loss and local inflammation
	Atherosclerosis	Reduced atherosclerosis
15-LO transfection	NTS-nephritis	Preserved glomerular functions
15-LO knockout mice	Cornea thermal injury	Reduced LXA_4 formation
		Defect in corneal reepithelialization
5-LO knockout mice	*T. gondii* infection	Reduced LXA_4 production
		Marked encephalitis
		Increased mortality
	M. tuberculosis infection	Elevated IL-12
		Reduced LXA_4 production
		Increased IL-12 and interferon-γ
		Enhanced resistance
COX-2 knockout mice	Acute lung injury	Increased inflammation
P-selectin knockout mice		Reduced LXA_4 production
	Anti-GBM nephritis	Enhanced PMN and albuminuria
Altered receptor and signaling		
Human ALX transgenic mice	Peritonitis	Decreased PMN infiltration
		Increased sensitivity to ATLa
	Acute lung injury	Blocked PMN recruitment
FPR knockout mice	Myocardial injury	ALX ligands (ATL and annexin I-derived peptide) retained cardioprotection
eNOS knockout mice; iNOS knockout mice	Peritonitis	Reduced anti-inflammatory action by aspirin and ATL

NTS, nephrotoxic serum; GBM, glomerular basement membrane.

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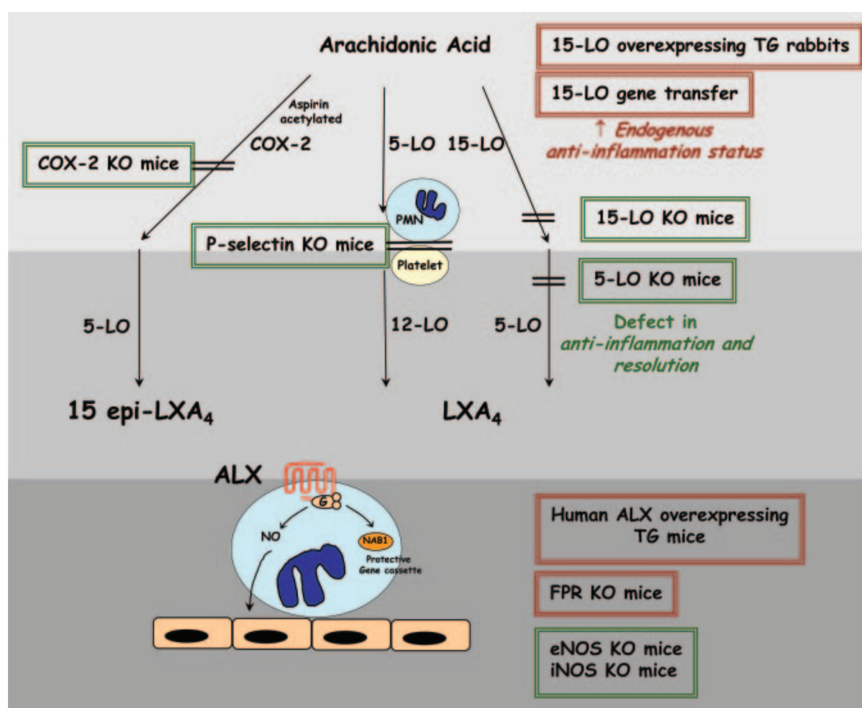

FIG. 6. In vivo evidence of the protective actions of LXA_4-ALX system: lessons learned from genetically engineered animals. Enhancement of either LXA_4 biosynthesis [i.e., 15-LO transgenic rabbits (Serhan et al., 2003) or 15-LO gene transfer (Munger et al., 1999)] or receptor expression [i.e., ALX transgenic mice (Devchand et al., 2003; Fukunaga et al., 2005)] give protection in inflammation. On the other hand, disruption of LXA_4 or ATL biosynthesis [i.e., 15-LO (Gronert et al., 2005), 5-LO (Aliberti et al., 2002b, Bafica et al., 2005), COX-2 (Fukunaga et al., 2005), and P-selectin knockout mice (Mayadas et al., 1996)] or ALX-dependent signaling [i.e., eNOS and iNOS knockout mice (Paul-Clark et al., 2004)] correlates with aberrant inflammatory responses. In FPR knockout mice, ALX ligands (i.e., LXA_4 and annexin I-derived peptide) retain their cardioprotective action, indicating that these ligands are selective for ALX and their bioactions do not involve FPR (Gavins et al., 2005).

dation and bone loss as well as the enhanced anti-inflammation status. Earlier results with these transgenic rabbits demonstrated protection from atherosclerosis (Shen et al., 1996). In view of the more recent results, the mechanism for this protection in 15-LO transgenic rabbits is probably related to the anti-inflammatory property of LXA_4 (Serhan et al., 2003). Along these lines, ALX was recently identified in mouse corneas, and topical treatment of LXA_4 increases the rate of reepithelialization and attenuates the sequelae of thermal injury (Gronert et al., 2005). Interestingly, 15-LO knockout mice (referred as mouse 12/15-LO or Alox15) exhibit a defect in corneal reepithelialization that correlates with a reduction in endogenous LXA_4 formation. These results identify a protective action for mouse 15-LO and LXA_4 in wond healing that now joins their well-recognized anti-inflammatory actions.

3. Counter-Regulatory Role for 5-Lipoxygenase in Lipoxin A_4 Biosynthesis. As noted above, LXA_4 and ATL exert a regulatory role on dendritic cell IL-12 production stimulated by *T. gondii* extract (Aliberti et al., 2002b). Interestingly, *T. gondii*-exposed wild-type, but not 5-LO knockout, mice produce high levels of LXA_4 at the onset of chronic infection (Aliberti et al., 2002b). In this model, 5-LO knockout mice display marked encephalitis and increased mortality associated with a significant elevation of IL-12, which is not likely to be attributable to the reduced level of LXA_4 in these mice. More recent findings with 5-LO knockout mice also demonstrate that diminished LXA_4 production is associated with enhanced splenocyte recall responses and decreased bacterial burdens in lungs during *Mycobacterium tuberculosis* infection, establishing LXA_4 as a key mediator in resistance to *M. tuberculosis* infection (Bafica et al., 2005). These results indicate a novel role for LXA_4 pathway in regulating microbial infection.

4. Lipoxin A_4 in Other Knockout Mouse Models. A major disruption in resolution was demonstrated in P-selectin knockout mice. P-selectin on the surfaces of activated endothelium and platelets mediates PMN-endothelial as well as PMN-platelet interactions in vivo that are essential for LXA_4 generation via transcellular biosynthetic routes (see section II. and Fig. 1). In an acute passive antiglomerular basement membrane nephritis, a 2-fold increase in glomerular PMNs and albuminuria is observed in P-selectin knockout mice. This aberrant inflammation is associated with a 60% reduction of LXA_4 and highlights an important role of cell-cell interaction and LXA_4 formation in regulating inflammation (Mayadas et al., 1996). In acute pleuritis, aspirin increased plasma NO, which correlated with a reduction in inflammation. Both aspirin and ATL inhibited leukocyte trafficking in a NO-dependent manner, and this effect is abolished in either constitutive (eNOS) or inducible NO synthase (iNOS) knockout mice compared with wild-type mice (Paul-Clark et al., 2004). These re-

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sults demonstrate that aspirin evokes vascular ATL generation, which in turn stimulates local NO production that participates in anti-inflammatory circuits in vivo. Recently in a spontaneously resolving model of acute lung injury (ALI), both selective pharmacologic inhibition and gene disruption of COX-2 block resolution of ALI (Fukunaga et al., 2005). These findings indicate a protective role in ALI for COX-2-derived mediators (e.g., ATL), in part via enhanced LX signaling, and carry potential therapeutic implications for this devastating clinical disorder.

VIII. How Does Lipoxin A_4 Induce Anti-Inflammatory and Proresolving Signaling?

A. Ligand and Receptor Dependence: Direct Functional Links between Lipoxins and ALX

LXA_4, ATL, and their stable analogs in vivo activate endogenous anti-inflammation and accelerate resolution, supporting the concept that lipoxins serve as agonists for ALX. Their protective actions are ALX-dependent based on the following evidence.

1. Blockade in Vitro. Differentiated HL-60 cells exposed to an ALX antisense oligonucleotide selectively lost [^{3}H]LXA_4 binding as well as LXA_4-stimulated lipid remodeling that paralleled the loss of mRNA for ALX (Fiore and Serhan, 1995). In peripheral blood monocytes, the antipeptide antibody to ALX blocks LXA_4-induced calcium mobilization (Maddox et al., 1997).

2. Overexpression in Vitro and in Vivo. Recently, a reporter gene assay using HEK293 cells transiently transfected with ALX was established. This system permits the direct assessment of TNF-α induced NF-κB activation and regulation by recombinant ALX interactions with the ligand. Recombinant human ALX potently inhibits NF-κB activation only in the presence of ligand ATLa (Devchand et al., 2003). In addition, transgenic mice overexpressing human ALX display a profound anti-inflammatory phenotype, with markedly decreasing PMN infiltration with endogenous LXA_4. Moreover, these human ALX mice show increased sensitivity in response to the suboptimal doses of exogenous ATLa in vivo, shifting the dose-response curve to the left compared with their nontransgenic littermates. These results provide compelling evidence for direct functional links between LXA_4 and functional roles for human ALX in vivo (Devchand et al., 2003). In the CHO cell system, ATL directly triggered concentration- and time-dependent tyrosine phosphorylation of selective proteins in an ALX-dependent manner (N. Chiang and C. N. Serhan, manuscript in preparation).

3. Independent Proof. Recent results provided by several independent groups further demonstrate that overexpression of ALX in vitro amplified the action of LXA_4. For example, Kucharzik et al. (2003) showed that overexpression of ALX in intestinal epithelial cells conferred enhanced down-regulation of IL-8 expression in response to the ligand LXA_4. This action is particularly relevant because the intestine is highly colonized with bacteria and presumably high levels of fMLF. However, the intestine is not in a constant inflamed state, and the intestinal epithelia constitutively is not activated by fMLF, further supporting the fact that fMLF is not a physiologic ligand for the receptors of the FPR family. Sodin-Semrl et al. (2004b) also constructed a CHO cell line stably expressing ALX together with a human IL-8 promoter luciferase gene to determine the role of NF-κB in IL-8 gene regulation. In this recombinant system, the NF-κB pathway proved to be preeminent for the biologic responses elicited by LXA_4. Moreover, Vaughn et al. (2002) reported that LXA_4 stimulates production of inositol triphosphate in COS-1 cells cotransfected with mouse ALX and Gα16. Wu et al. (2006) demonstrated that LXA_4 down-regulates the effects of CTGF on chemokine release and phosphorylation of MAPK, phosphatidylinositol 3-kinase, and Akt in cultured mesangial cells. Each of these actions is demonstrated to be ALX-dependent since transfection of mouse ALX into the cells intensified the inhibitory action of LXA_4 in response to CTGF.

Note that the accessibility of some of these reagents, including ATLa and radiolabeled LXA_4 and ATLa, is limited because they were not made commercially available. Nevertheless, the fact that the reagents have limited availability in the field does not diminish the rigor of the results. In fact, multiple independent investigators each documented direct evidence providing further support that LXA_4 transmits stop signals to attenuate proinflammatory signals in an ALX-dependent manner with selective cell types both in vitro and in vivo. Moreover, recently non-LX small molecule mimetics were synthesized that act as ALX agonists and are anti-inflammatory in vivo in murine dermal inflammation (Burli et al., 2006).

B. In Vitro Cell Type-Specific Anti-Inflammatory Signals

The anti-inflammatory action of LXA_4 in vivo is likely to be a summation of multiple ALX-initiated signaling events in several cell types engaged in inflammation (Table 8; Fig. 7A).

1. Leukocytes: Polymorphonuclear Neutrophils, Monocytes, and Macrophages. LXA_4 displays differential actions on several types of leukocytes. In human PMN, LXA_4 blocks PMN transmigration. In monocytes, LXA_4 stimulates chemotaxis and adherence, but no apparent pro-inflammatory responses of these cells in vitro and in vivo were reported (Maddox et al., 1997; Hachicha et al., 1999). In this context, both LXA_4 and ATL stimulate the uptake of apoptotic PMN by monocyte-derived macrophages in a nonphlogistic fashion (Godson et al., 2000). This action seems to be coupled to changes in the actin cytoskeleton in macrophages (Maderna et al., 2002). These findings highlight the action of LXA_4 and ATL

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TABLE 8
Human ALX signal transduction during LXA_4 and ATL activation

The arrows indicate either increased (↑) or decreased (↓) activity.

Cell Type	G Protein Class	LXA_4 and ATL-Evoked Signals	Kinase Associated	Gene Expression	Up-Regulated by	References
Human HL-60 (differentiated)	G_i	↑ PLD activation	Protein kinase C		Retinoic acid, DMSO, PMA	Fiore et al. (1993)
Human PMN	G_i	↑ PLD activation; ↑ arachidonic acid release; ↑ PSDP; ↓ phosphorylation of LSP-1; no increase of cAMP, proton efflux, and $[Ca^{2+}]_i$	Tyrosine kinase; P38-MAPK	↑ NAB1; ↓ IL-8, AP-1, NF-κB		Nigam et al. (1990); Fiore et al. (1992); Levy et al. (1999); Qiu et al. (2001); Ohira et al. (2004)
Human monocyte	G_i	↑ $[Ca^{2+}]_i$; no increase of cAMP and proton efflux				Maddox et al. (1997)
Human T cells		↓ ERK activation				Ariel et al. (2003)
Human enterocyte		No proton efflux		↓ IL-8; ↓ NF-κB	IL-13, IL-4, interferon-γ	Gronert et al. (1998); Gewirtz et al. (2002); Kucharzik et al. (2003)
Human synovial fibroblast		↑ PLD activation; ↓ NF-κB binding		↑ TIMP-1 and TIMP-2; ↓ IL-6, IL-8, MMP-1, MMP-3	IL-1β, TGF-β	Sodin-Semrl et al. (2004a,b)
CHO expressing human ALX	G_i	↑ arachidonic acid release; no increase of cAMP and $[Ca^{2+}]_i$				Fiore et al. (1994); Sodin-Semrl et al. (2004b)
HEK293 expressing ALX		↓ NF-κB activity				Devchand et al. (2003)

DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor-β.

action in promoting resolution of inflammation since reduction in the numbers of PMNs, recruitment of monocytes to the sites of inflammation and injury, and clearance of apoptotic PMNs by macrophages are all the key events at the tissue level in wound healing and resolution. In addition, LXA_4 inhibits eosinophil chemotaxis in vitro in the nanomolar range (Lee et al., 1989; Bandeira-Melo et al., 2000a) and blocks human natural killer cell cytotoxicity in a stereoselective fashion (Ramstedt et al., 1987) and stimulates myeloid bone marrow-derived progenitors (Stenke et al., 1991). More recently, in activated human T cells ATLa inhibited TNF-α secretion by blocking ERK activation, indicating a role for LXA_4 and ATL in mediating T cell-mediated responses (Ariel et al., 2003). Thus, LXA_4 and ATL are also potential counter-regulatory signals in communication(s) between innate and acquired immune systems.

2. Epithelial Cells: Down-Regulation of Proinflammatory Genes. Epithelial cells of the alimentary tract play a central role in mucosal immunophysiology. In human enterocytes, direct ALX activation by LXA_4 and ATLa diminishes *Salmonella typhimurium*-induced IL-8 transcription (Gewirtz et al., 1998). The reduction of the IL-8 mRNA level parallels decrements in IL-8 secretion, indicating that in these cells the mechanism of action of ALX for blocking this chemokine is at the gene transcriptional level. In an effort to elucidate the mechanism by which these lipid mediators modulate cellular proinflammatory programs, global epithelial gene expression was surveyed using microarray analysis. ATLa pretreatment attenuates induction of ~50% of *Salmonella typhimurium*-induced gene expression (Gewirtz et al., 2002). A major subset of genes whose induction is reduced by ATLa is regulated by NF-κB, suggesting that ATLa is influencing the activity of this transcription factor. At a low nanomolar range, ATLa reduces NF-κB-mediated transcriptional activation in an ALX-dependent manner and inhibits degradation of IκBα. ALX is identified and cloned from human enterocytes under control of cytokines, of which lymphocyte-derived IL-13 and interferon-γ are the most potent (Gronert et al., 1998). In polarized T84 intestinal epithelial cells, ALX is preferentially expressed on the basolateral membrane of these cells when monitored by cell surface-selective biotinylation and confocal microscopy. In addition, overexpression of ALX enhances down-regulation of IL-8 expression in response to ATLa, giving direct functional links to the ALX receptor. Thus, LXA_4 generated in or near the paracellular space during neutrophil-epithelial interactions could rapidly act on epithelial ALX to down-regulate epithelial promotion of intestinal inflammation (Kucharzik et al., 2003). Along these lines, microarray analysis also revealed that epithelial cells of different origin express bactericidal/permeability-increasing protein, an antibacterial and endotoxin-neutralizing molecule that is transcriptionally regulated by ATLa (Canny et al., 2002).

3. Fibroblast: Inhibition of Proinflammatory Cytokines. ALX that is up-regulated by IL-1β and transforming growth factor-β was also identified in human fibroblast-like synoviocytes (Sodin-Semrl et al., 2004a,b). ALX expression in these cells modulated IL-6

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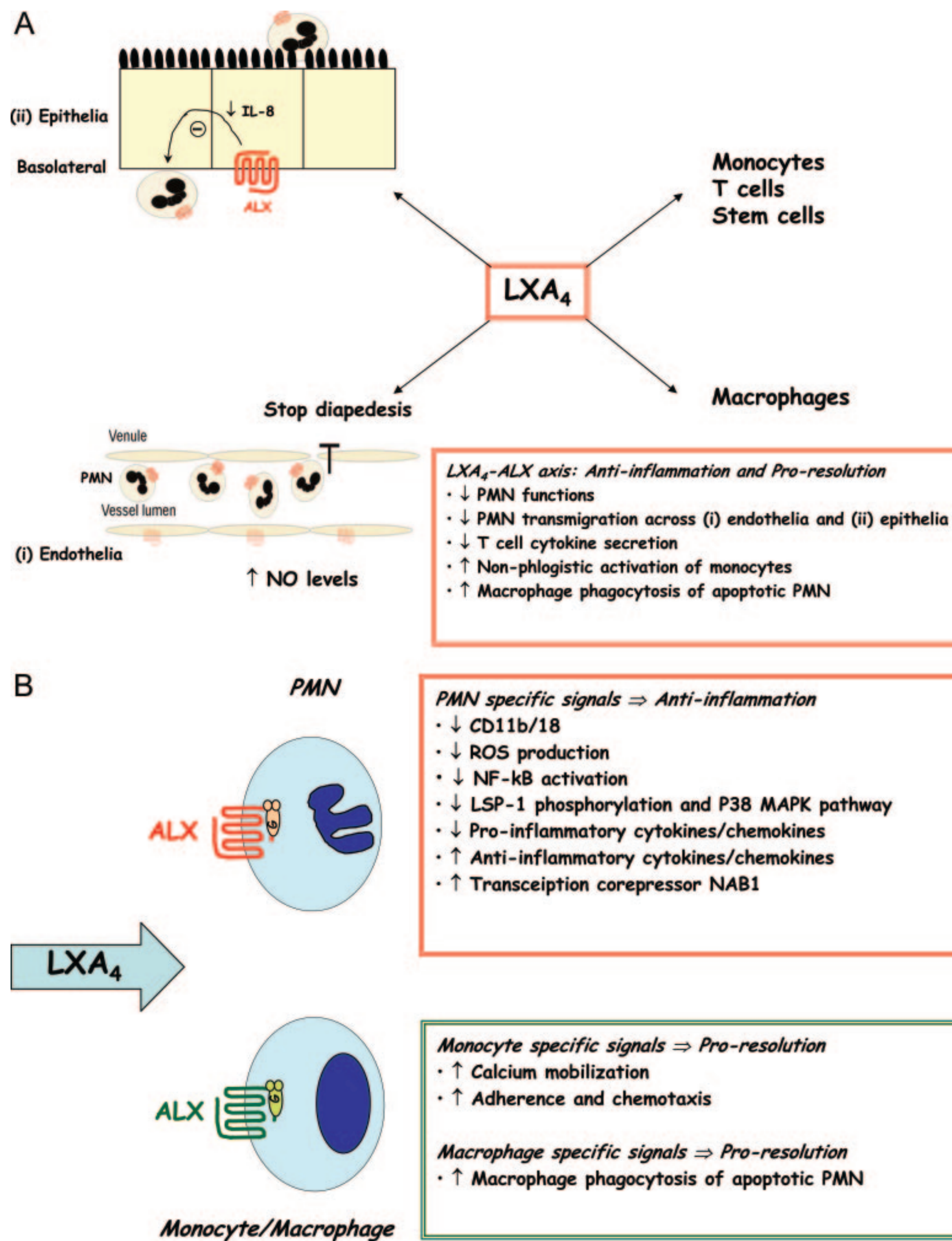

FIG. 7. LXA_4-ALX axis during multicellular interactions in inflammation: cell type-specific action. A, on the tissue level, the anti-inflammatory and proresolving action of LXA_4 is likely to be a summation of LXA_4-ALX interactions on multiple cell types engaged in inflammation. B, on the cellular level, LXA_4 exhibits distinct ALX-initiated signaling events on PMNs versus monocytes/macrophages. ROS, reactive oxygen species.

and matrix metalloproteinase (MMP)-1 and MMP-3 expression, implicating a regulatory mechanism of LXA_4 in pathogenesis of rheumatoid arthritis. In human synovial fibroblasts, LXA_4 inhibits IL-1β responses with reduction of IL-6 and IL-8 synthesis and prevents IL-1β-induced MMP-3 synthesis at nanomolar concentrations (Sodin-Semrl et al., 2000). In addition, LXA_4 induces increases in tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 proteins. Thus, LXA_4 and ATL regulate gene expression in synovial fibroblasts, an action apparently not shared by the peptide ligands of ALX. These findings suggest that LXA_4 may be involved in a negative feedback loop of cytokine-induced activation of synovial fibroblasts.

C. Leukocyte-Specific Intracellular Signals

1. Distinct ALX-Initiated Cellular Events. Lipoxin A_4 exhibits distinct ALX-initiated signaling events on PMNs versus monocytes/macrophages (Fig. 7B).

a. Polymorphonuclear neutrophils. ALX interaction with LXA_4 and ATL on PMNs regulates the polyisoprenyl phosphate signaling pathway (Levy et al., 1999) (Table 8). ALX activation reverses LTB_4-initiated polyisoprenyl phosphate remodeling, leading to accumula-

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tion of presqualene diphosphate, a potent negative intracellular signal in PMNs that inhibits recombinant phospholipase D and superoxide anion generation. In addition, LXA_4 reduces peroxynitrite formation and thus can oppose peroxynitrite signaling in leukocytes (Jozsef et al., 2002). More recently, ATL was reported to inhibit leukocyte trafficking in a NO-dependent manner on IL-1β-stimulated mouse mesentery (Paul-Clark et al., 2004). In retinoic acid-differentiated HL-60 cells, LXA_4 stimulates phospholipase D activation that is staurosporine-sensitive, suggesting the involvement of protein kinase C in signal transduction in these cells (Fiore et al., 1993). LXA_4 also down-regulates IP_3 generation (Grandordy et al., 1990) and CD11b/18 and blocks LTB_4 or fMLF-stimulated PMN transmigration or adhesion by regulation of β2-integrin-dependent PMN adhesion (Fiore and Serhan, 1995). This modulatory action is partially reversed by prior exposure to genistein, a tyrosine kinase inhibitor (Fiore and Serhan, 1995). Along these lines, using proteomic analysis, ATLa blocks phosphorylation and activation of leukocyte-specific protein-1 along with several other components of the p38 MAPK pathway in human PMNs. These results indicate a regulatory role for LXA_4 and ATL on the p38 MAPK cascade, which is known to promote chemotaxis and other inflammatory responses (Ohira et al., 2004).

b. Monocytes. LXA_4 triggers intracellular Ca^{2+} release and adherence to laminin in human peripheral blood monocytes and cultured THP-1 cells (Romano et al., 1996; Maddox et al., 1997). Interestingly, LXA_4-stimulated monocyte adherence to laminin is not dependent on the increase in $[Ca^{2+}]_i$ since a Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) does not change the LXA_4-stimulated adherence response (Romano et al., 1996). Furthermore, the EC_{50} value for the LXA_4-stimulated increase in $[Ca^{2+}]_i$ is >100 nM in monocytes, which is >2 log orders of magnitude higher than that required for LXA_4-stimulated adherence (EC_{50} <1 nM). Although LXA_4 stimulates calcium mobilization in monocytes, Ca^{2+} is not the second messenger of LXA_4 actions in monocytes. LXA_4 stimulates chemotaxis and adherence in monocytes but no apparent “proinflammatory” responses of these cells in vitro or in vivo. Thus, different intracellular signaling pathways are present in PMNs versus monocytes despite identical receptor sequences. In view of G protein-coupling events in monocytes, both Ca^{2+} mobilization and adherence are PTX-sensitive. This indicates that receptor coupling in monocytes and PMNs is similar on this basis. GPCRs are well known to couple to different G proteins and/or have different signaling pathways in different cells (Kenakin, 2002), especially in comparisons between natural and recombinant systems (Kenakin, 1997). Thus, there could be different PTX-sensitive G protein subtypes that couple to the intracellular domains of ALX with divergent downstream signal transduction pathways in different cell types leading to, for example, chemotaxis of monocytes versus reduction of PMNs (Maddox et al., 1997; Chiang et al., 2000).

2. Gene Regulation. LXA_4-ALX activation regulates both proinflammatory and protective gene cassettes. By using differential display reverse transcription-polymerase chain reaction, a subset of genes was selectively up-regulated in human PMNs upon short exposure to ATLa. Among them, a transcriptional corepressor NAB1 identified previously as a glucocorticoid-responsive gene in hamster smooth muscle cells was further investigated and also found to be up-regulated by ATLa in murine lung vascular smooth muscle in vivo (Qiu et al., 2001). These findings provide evidence for rapid transcriptional induction of a cassette of genes via an ATLa-stimulated GPCR pathway. In addition, ATLa attenuates nuclear accumulation of AP-1 and NF-κB in both PMN and monocytes and inhibits IL-8 mRNA expression (Jozsef et al., 2002).

IX. Additional Receptors Involved in Lipoxin A_4 and Aspirin-Triggered Lipoxin A_4 in Vivo Actions

A. Cysteinyl-Leukotriene Receptors

Results from pharmacologic experiments (Fig. 8) indicate that LXA_4 also acts via interactions with a subclass of peptido-LT receptors ($CysLT_1$) as a partial agonist to mediate the bioactions in several tissues and cell types other than leukocytes (Badr et al., 1989; Fiore et al., 1994). LXA_4 (in the nanomolar range) blocks LTD_4 actions and also competes for specific $[^3H]LTD_4$ binding on mesangial cells (Badr et al., 1989) and human umbilical vein endothelial cells (HUVECs) (Fiore et al., 1993; Takano et al., 1997). HUVECs specifically bind $[^3H]LXA_4$ with a K_d of 11 nM, which can be inhibited by LTD_4 and SKF104353 (Fiore et al., 1993). Therefore, LXA_4 interacts with at least two classes of cell surface receptors: one specific for LXA_4, which is present on leukocytes and enterocytes (ALX), and the other shared by LTD_4, which is present on HUVECs and mesangial cells ($CysLT_1$) (Norel and Brink, 2004).

An inducible form of $CysLT_1$ was identified and cloned from HUVECs (Gronert et al., 2001). This recombinant $CysLT_1$ receptor gives stereospecific binding with both $[^3H]LTD_4$ and the labeled mimetic of ATL ($[^3H]$ATLa; 15-epi-16-phenoxy-*para*-fluoro-LXA_4), which are displaced with LTD_4 and ATLa (~IC_{50} = 0.2–0.9 nM) and not with a biologically inactive ATL/LXA_4 isomer. In contrast, LTD_4 is an ineffective competitive ligand for recombinant human ALX with $[^3H]$ATLa. Endogenous murine $CysLT_1$ receptors also give specific $[^3H]$ATLa binding that is displaced with essentially equal affinity by LTD_4 or ATLa. Systemic ATLa proves to be a potent inhibitor (>50%) of $CysLT_1$-mediated vascular leakage in murine skin (200 μg/kg) in addition to blocking PMN recruitment into dorsal air pouches (4 μg/kg). These

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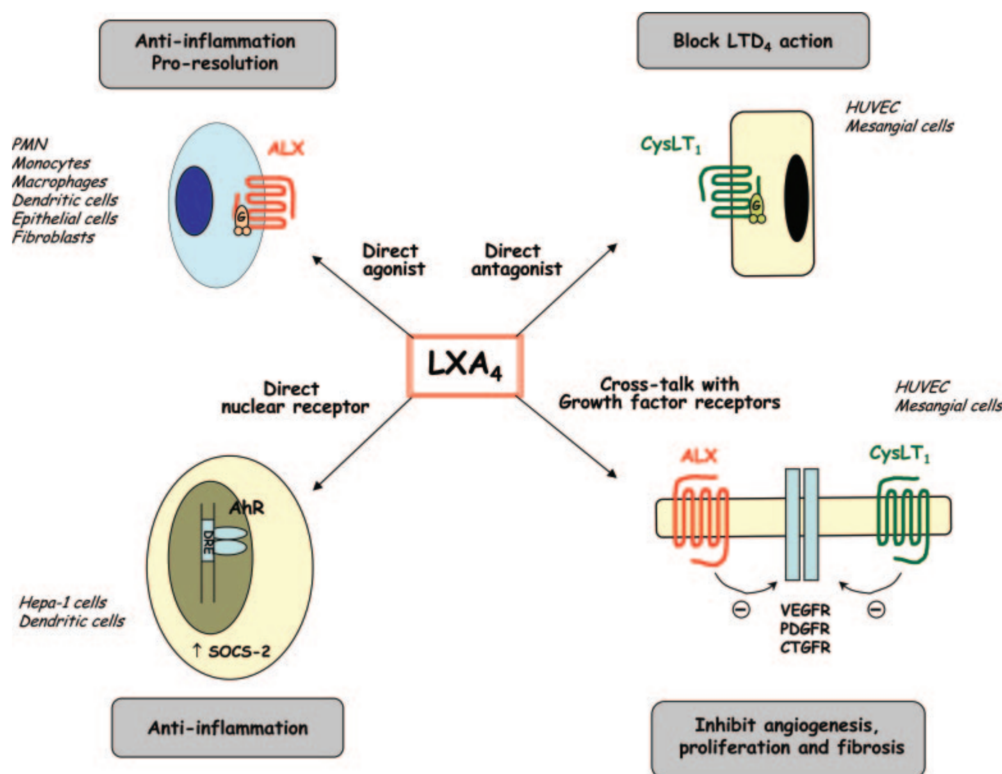

FIG. 8. The anti-inflammatory action of LXA_4 is mediated by multiple receptors in vivo. The overall action of LXA_4 in vivo is likely to be attributed to its interactions with multiple receptors, including 1) direct activation of ALX as a receptor agonist, 2) direct inhibition of $CysLT_1$ as a receptor antagonist, 3) direct activation of the nuclear receptor AhR, and 4) cross-talk with growth factor receptors. DRE, dioxin response element; suppressor of cytokine signaling-2 (SOCS-2).

results indicate that ATL and LTD_4 bind and compete with essentially equal affinities at $CysLT_1$, providing a molecular basis for ATL serving as a local damper of both vascular $CysLT_1$ signals as well as ALX-regulated PMN trafficking.

In human renal mesangial cells, LXA_4 inhibits PDGF and LTD_4-stimulated proliferation via regulation of PDGF receptor β (McMahon et al., 2002). In addition, LXA_4 stimulates the MAPK superfamily via two distinct receptors: one via a PTX-sensitive G protein, leading to p38 activation, and the other via a PTX-insensitive G protein, leading to ERK activation (McMahon et al., 2000). The modulation of MAPK activities by LXA_4 with mesangial cells is PTX-insensitive (McMahon et al., 1998), suggesting the potential presence of additional LXA_4 receptor subtypes and/or signaling pathways (Maderna et al., 2000). Thus, besides $CysLT_1$, LXA_4 may also interact with $CysLT_2$ and/or additional CysLT receptors that have not yet been described.

B. Ah Receptor

Another potential receptor mediating LXA_4 action in vivo is the Ah receptor (AhR) (Schaldach et al., 1999), a ligand-activated transcription factor that mediates many of the biologic actions of a large class of environmental compounds. LXA_4 binds to and activates AhR in Hepa-1 cells. LXA_4 competes for specific [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (an AhR ligand) binding with an EC50 of 100 nM and transforms the AhR to an active dioxin response element-binding form in a concentration-dependent manner. Along these lines, the most recent findings by Machado et al. (2006) demonstrated that LXA_4 activates two receptors in dendritic cells, ALX and AhR. This activation triggers expression of suppressor of cytokine signaling-2. These results indicate that AhR can also function as a LXA_4-specific nuclear receptor in vivo together with ALX.

C. Growth Factor Receptors: Cross-Talk with ALX and/or CysLT$_1$

ATLa inhibits vascular endothelial growth factor (VEGF)-stimulated endothelial cell proliferation and chemotaxis in vitro and angiogenic phenotype in vivo (Fierro et al., 2002). Because it is reported that human endothelial cells express ALX (Koczulla et al., 2003), it is likely that the antiangiogenic action of ATL is mediated by ALX cross-talking with the VEGF receptor. ATL also regulates other receptors of the growth factor family. For example, LXA_4 down-regulates the effects of CTGF on chemokine release and phosphorylation of MAPK, phosphatidylinositol 3-kinase, and Akt in cultured mesangial cells in an ALX-dependent manner (Wu et al., 2006). In this context, ATL blocks PDGF-stimulated proliferation via inhibition of tyrosine phosphorylation of PDGF receptor β on human renal mesangial cells, an action that probably involves both ALX and $CysLT_1$

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(McMahon et al., 2002). Similarly, $CysLT_1$ has also been recently demonstrated to trans-activate epidermal growth factor receptors in human airway smooth muscle cells to induce proliferation (Ravasi et al., 2006). Together, these results highlight the complex cross-talk between two families of receptors: 1) GPCRs (e.g., ALX and $CysLT_1$) and 2) growth factor receptors (e.g., VEGF, PDGF, and CTGF receptors) to control proliferation, angiogenesis, and fibrosis, an action that now joins the repertoire of LX bioactions in the inflammatory milieu.

1. Which of These Molecules Mediate Lipoxin A_4 Actions in Vivo? It is likely that the overall action of LXA_4 in vivo is attributed to 1) receptors and 2) ligands. Each of these receptors, ALX, $CysLT_1$, and Ah, can contribute to the bioactions of LXA_4 in vivo. The rank order of importance depends on where and when these different types of receptors are expressed. The exogenous (pharmacologic) addition of the ligand LXA_4 at high concentrations (high nanomolar to micromolar) may activate each of these receptors (ALX, $CysLT_1$, and Ah) with robust cellular responses. In comparison, the ligand LXA_4, when produced endogenously (physiological) in low amounts (picomolar to low nanomolar) within a local environment (organ or tissue) may only have access to selective types of receptors, evoking highly specific and tightly controlled signals to modulate innate and acquired immune responses.

X. Conclusions

Here we summarize current observations and results concerning the GPCR denoted as ALX (formally FPRL1). The molecular characterization of ALX, and in vitro and in vivo evidences clearly indicate the following:

- The lipid-derived mediators LXA_4 and ATL display stereoselective and high-affinity specific binding to ALX and transduce receptor-dependent signaling cascade(s).
- These agonists are generated endogenously and exhibit highly potent protective actions (low to subnanomolar range) both in vitro and in vivo.
- The interactions of LXA_4 and ATL with ALX are absolutely stereospecific in that the structure requirements of LXA_4 and ATL for receptor activation are also essential for their in vivo protective actions.
- Although the nucleotide sequence of ALX/FPRL1 is ~70% identical to that of FPR, most ALX peptide ligands require micromolar levels in vivo to evoke ALX-dependent responses.

Taken together, the bioactive endogenous mediator LXA_4 and the aspirin-triggered epimer ATL are the most potent and selective agonists documented to date for ALX. They establish the important contribution of this ligand-receptor pair in in vivo action of LXs. These results do not, however, rule out the involvement of additional and yet to be discovered receptors that can also contribute to LX action in vivo. The identification of these newly appreciated endogenous anti-inflammatory circuits (i.e., LXA_4 and ALX) offers "agonist-driven" molecular mechanism(s) for therapeutic approaches and disease interventions. This notion contrasts with the traditional approaches that rely heavily on the development and use of inhibitors of biosynthetic pathway(s) and/or receptor antagonists of inflammatory mediators. A comprehensive understanding of the endogenous pathways and cellular mechanisms that counter-regulate inflammatory responses and promote natural resolution phase is required (Bannenberg et al., 2005) to uncover the currently unappreciated sides of resolution biology and pathogenesis of human diseases that might be useful in developing new drugs to treat inflammatory disorders.

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