THE ROLE OF LTB$_4$-BLT1 AXIS IN CHEMOTACTIC GRADIENT SENSING AND DIRECTED LEUKOCYTE MIGRATION

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Abstract

Directed leukocyte migration is a hallmark of inflammatory immune responses. Leukotrienes are derived from arachidonic acid and represent a class of potent lipid mediators of leukocyte migration. In this review, we summarize the essential steps leading to the production of LTB$_4$ in leukocytes. We discuss the recent findings on the exosomal packaging and transport of LTB$_4$ in the context of chemotactic gradients formation and regulation of leukocyte recruitment. We also discuss the dynamic roles of the LTB$_4$ receptors, BLT1 and BLT2, in mediating chemotactic signaling in leukocytes and contrast them to other structurally related leukotrienes that bind to distinct GPCRs. Finally, we highlight the specific roles of the LTB$_4$-BLT1 axis in mediating signal-relay between chemotaxing neutrophils and its potential contribution to a wide variety of inflammatory conditions including tumor progression and metastasis, where LTB$_4$ is emerging as a key signaling component.

Keywords

leukotriene B$_4$; neutrophils; chemotaxis; inflammation; migration

INTRODUCTION

Cell migration is a fundamental process conserved from bacteria to humans. Migration of cells towards a chemical cue, referred to as directed cell migration or chemotaxis, contributes to a number of fundamental biological responses in different organisms. For example, bacteria use chemotaxis to interact with chemoattractant-releasing plant roots to establish a symbiotic relationship [1] and in the case of *H. pylori*, which causes stomach ulcers and gastric cancers, chemotactic signaling is required to establish stomach colonization by the bacteria [2]. The social amoeba, *Dictyostelium discoideum*, upon nutrient depletion chemotax to form cellular aggregates that then differentiate into multicellular slugs and fruiting bodies [3]. In higher organisms including flies, zebrafish and mouse, directed migration of primordial germ cells is observed very early during development [4]. Finally, leukocyte migration in response to inflammation is another striking example of chemotaxis in higher mammals, including humans [3].

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The chemical nature of chemoattractants varies widely from small molecules and lipids to peptides and proteins depending on the model system. *E. coli* chemotax to many chemicals including glucose and amino acids such as aspartate and serine [5]. For *Dictyostelium*, both folic acid and cyclic adenosine monophosphate (cAMP) act as physiological chemoattactants [3]. Not surprisingly, in higher eukaryotes, the complexity of chemoattractants is impressive and ranges from formylated peptides secreted by bacteria (such as N-formylmethionyl-leucyl-phenylalanine - fMLP), to products of the complement cascade (such as C5a) and phospholipid metabolites (such as leukotriene B_4 (LTB_4)) as well as the large family of chemokines that are derived from endothelial, epithelial, and stromal cells [6, 7]. Chemoattractants are further considered to be either end-point/primary (fMLP, C5a *etc.*) or intermediate/secondary (LTB_4, IL8 *etc.*), depending on their chemotactic potential in competing gradients and signaling specificity [8], an aspect that will be discussed later. Remarkably, despite their diverse nature, the vast majority of chemoattractants bind and activate specific G-protein coupled receptors (GPCRs) to mediate their effects [3, 6].

For cells to chemotax in complex *in vivo* environments, directional cues need to be sustained for significant period of time over long distances. One of the ways cells accomplish sustained long-range chemotactic behavior is by 'signal-relay'. For example, in *Dictyostelium* cAMP induces further production of cAMP through activation of Adenyl cyclase A or ACA, which then promotes and sustains a head-to-tail chemotactic response of migrating cells [9–11]. In the case of immune cells such as neutrophils, signal-relay occurs when cells chemotax toward a primary chemoattractant source, where they then actively produce and secrete a secondary chemoattractant that is required to drive the directional movement of both the cells that produce it (autocrine signaling) as well as the cells that follow it (paracrine signaling) in a spatio-temporal manner [9, 12–15]. In this review, we will specifically focus on the production, gradient formation and regulation of signal-relay by the interaction of LTB_4 with its GPCRs - BLT1 and BLT2, with an emphasis on the role of such an axis in regulating chemotactic responses of immune cells in a variety of inflammatory scenarios, including cancer.

LTB_4 SYNTHESIS, REGULATION AND SECRETION

Before we dwell on the functional consequences of LTB_4-BLT interactions, it is essential to understand the sequence of events leading to synthesis, packaging and secretion/distribution of LTB_4 in the context of leukocyte activation by inflammatory agents.

Tissue distribution of LTB_4

In 1979, Borgeat and Samuelsson described the release of C20 dihydroxy fatty acids with three conjugated double bonds, first from rabbit [16, 17] and then from human morphonuclear leukocytes [17] in response to arachidonic acid (AA) and the calcium ionophore A23187. This class of eicosanoid was called LTB_4 to reflect the nature of the compounds as well as their source [18]. Since then, LTB_4 is known to be produced by tissues under various conditions, most notably during inflammatory responses.

LTB$_4$ production by immune cells

Leukotriene release from human mature myeloid cells occurs in response to stimulation with molecules including endotoxin, C5a, IL-1, TNFα, GMCSF, LIF and PAF. However, the quantity released varies among myeloid cell types. Basophils, for example, do not produce LTB$_4$, but can release a related leukotriene, LTC$_4$, upon stimulation with anti-IgE or A23187 [19]. Similarly, A23187-stimulated eosinophil primarily secrete LTC$_4$ and only very low quantities of LTB$_4$ [20]. In contrast, neutrophils primarily release LTB$_4$ upon stimulation [20]. On the other hand, human monocytes can produce both LTB$_4$ or LTC$_4$ in response to physiologically relevant stimuli such as unopsonized zymosan particles or IgG-sensitized sheep erythrocytes or to non-physiological stimulus like A23187, with a slight preference towards LTB$_4$ [21]. Similar observations have been reported for macrophages and mast cells [22]. However, mucosal mast cells isolated from the intestine of *Nippostrongylus brasiliensis*-infected rats and activated with anti-IgE show higher LTC$_4$ production rates compared to LTB$_4$ [23].

The production of LTB$_4$ from lymphocytes has been mottled in controversy. Although the production and release of LTB$_4$ from human T cells were initially reported [24], further work on highly purified T-lymphocytes and monoclonal lymphoblastoid cells failed to detect LTB$_4$, either in the presence or absence of AA or calcium ionophores [25]. Studies on T and NK cell lines such as Jurkat and RNK-16 also showed no evidence of LTB$_4$ release upon stimulation [26]. Similar reports on the absence of LTB$_4$ in B cells were first published in late 1980s and early 1990s but were quickly reconsidered after the demonstration of *in vitro* synthesis of LTB$_4$ from B cell lysates [27]. Later, B cells activated by the protein A-positive *Staphylococcal* strain, Cowan 1, and a mixture of recombinant IL-2/IL-6 were shown to possess high capacity to generate LTB$_4$ [27].

Elevated leukotriene secretion in myeloid cells, especially cells of myeloblastic origin, has important implications in the role of LTB$_4$ during immune surveillance and immune cell chemotactic responses. These cells either reside in tissues or actively survey injury and infection sites by detecting chemoattractant gradients. In later sections, we will describe how LTB$_4$ is especially well suited to amplify primary chemotactic signals by recruiting cells of lymphoid origin to inflammation sites.

LTB$_4$ production by non-immune cells

The other major source of LTB$_4$ in systemic circulation comes from non-immune sources associated with inflammatory pathophysiology. Asthma and upper respiratory airway inflammation represent the best-studied cases of LTB$_4$-related inflammatory pathophysiology. While neutrophils represent the major source of LTB$_4$ in bronchoalveolar lavage in bronchial asthma [28], a proportion of leukotrienes is produced by allergic lung alveoli that is involved in recruiting macrophages [29]. Also, LTB$_4$ release from tracheal epithelial cells during asthma has been shown to contribute to bronchial constriction and vasodilation [30]. Surprisingly, the brain is another major source of LTB$_4$ in non-immune tissues and LTB$_4$ synthesis positively correlates with aging in both rats [31] and humans [32]. The expression of 5-Lipoxygenase (5-LO), the enzyme responsible for LTB$_4$ production, in different parts of the rat brain, including the cerebellum and hippocampus, has

been reported and significant changes in LTB$_4$ levels have been associated with age-dependent cognitive impairment [33]. The expression of LTB$_4$ synthesizing enzymes in neurons and glial cells and the involvement of LTB$_4$ in brain ischemia, trauma, astrocytoma and other inflammatory pathophysiology of the brain provides indirect evidence for a role of LTB$_4$ in such conditions [34]. In fact, inhibition of LTB$_4$ production has been shown to be neuroprotective in cases of brain injury [35]. Finally, elevated LTB$_4$ levels have been found in a disparate array of tissues including corneal and conjunctival tissues of the eye [36], loose connective tissues such as adipocytes [37], endocrinal glands such as thyroid [38] and by mucosal epithelial cells in pathological conditions such as during ulcerative colitis [39] and type C gastritis [40].

Biosynthesis of LTB$_4$

The synthesis of LTB$_4$ begins with the mobilization of AA on the nuclear membrane, where free AA is released by the cleavage of the arachidonoyl ester from a membrane glycerophospholipid by the hydrolytic action of a calcium sensitive phospholipase A$_2$ (cPLA$_2$) [41]. The released AA then binds to an integral nuclear membrane protein called 5-Lipoxygenase activating protein (FLAP) [42]. Sequestration of AA by FLAP increases local AA concentration which changes membrane anisotropy and fluidity, a key step for the recruitment of 5-LO to the nuclear membrane [43, 44]. Once recruited, 5-LO interacts with FLAP, most likely through the interaction of the α2 domain of FLAP [45] and the dimerization interface of 5-LO [46], to further anchor it to the membrane. FLAP then delivers the substrate AA to 5-LO and through the action of 5-LO, AA then undergoes hydrogen abstraction at the C7 position followed by the addition of an oxygen to give rise to 5-hydroperoxyeicosatetraenoic acid (5-HpETE). This step is followed by the removal of the hydrogen atom from the C10 position to form a conjugate triene epoxide LTA$_4$ [47]. LTA$_4$ is then acted upon by LTA$_4$ hydrolase (LTA$_4$H) to give rise to LTB$_4$ by the addition of a water molecule at the C12 position [48]. Apart from the catalytic processes involved, another level of regulation of leukotriene synthesis occurs via post translation modifications, calcium binding and, more importantly, by the subcellular partitioning of the enzymes involved in LTB$_4$ synthesis, which will be discussed further below.

Regulation of cPLA$_2$

The key enzyme that connects cell stimulation with the initiation of leukotriene synthesis is cPLA$_2$, which can be activated by binding of growth factors, mitogens, cytokines, formylated peptides to their cognate receptors, as well as non–receptor mediated triggers such as oxidative and shear stress. The common factor between these disparate processes is the increase in cytosolic calcium either from emptying of internal stores or through extracellular calcium influx. The increase in cytosolic calcium is required for cPLA$_2$ to bind to membranes [49] and causes cPLA$_2$ to rapidly redistribute from the cytosol to the endoplasmic reticulum (ER) and the nuclear envelope where it hydrolyzes nuclear phospholipids to release AA. Additionally, cell activation leads to rapid phosphorylation of cPLA2 resulting in its increased lipase activity (Fig.1) [50] [51]. While phosphorylation by MAPK [52] and CAM kinases [53] are thought to mediate translocation, other reports suggest effects on enzyme activity alone [54]. *Hence, the precise role of cPLA2 phosphorylation in its nuclear translocation remains controversial.* Interestingly, cPLA$_2$

phosphorylation is dependent on the type of stimuli: in neutrophils both ERK and p38MAPK phosphorylate cPLA$_2$ in response to PAF, but not fMLP [55].

Regulation of FLAP

The modulation of AA availability by FLAP is one of the least understood steps in the leukotriene synthesis cascade. FLAP belongs to a protein superfamily called membrane-associated proteins involved in eicosanoid and glutathione metabolism, or MAPEG. But unlike other members of the same family, such as LTC$_4$ synthase, FLAP does not possess any inherent enzymatic activity [56] and the mechanisms by which FLAP binds to AA in the nuclear lipid bilayer and how it transfers AA to 5-LO are not well understood. It is well established that 5-LO nuclear translocation and AA catalysis are independent of FLAP. Also, there is no literature to suggest that calcium binding to the C2-like domain of 5-LO in anyway mediates the direct interaction of FLAP with 5-LO. However, FLAP is essential for the transfer of AA to 5-LO, as LTB$_4$ synthesis fails to occur when cells are treated with a specific competitive inhibitor of FLAP, MK886. The regulation of LTB$_4$ synthesis by FLAP is perhaps mediated by a "catch and release" mechanism of AA to 5-LO [57]. According to the recently solved inhibitor bound crystal structure of FLAP [45], free AA molecules laterally diffuse within the plane of the membrane and bind in the three hydrophobic surface grooves formed by the FLAP trimer. AA-bound FLAP then undergoes a conformational change in its positively charged cytosolic loops allowing interaction with 5-LO. The mechanism by which the "caught" AA is now "released" to 5-LO is not understood. Interestingly, mutations in the cytosolic loops of FLAP, the regions that are proposed as 5-LO interaction sites, have marked effect on binding affinities of FLAP to AA, suggesting that these flexible loops are central to the "capture" and "release" of AA. Moreover, FLAP has been shown to exist as a homodimer [58] and a trimer [45] or even as a multimer [59], but whether FLAP multimer formation is ligand-induced and whether such arrangement is required for its function remains unresolved.

Regulation of 5-LO

5-LO is a non-heme iron-containing enzyme that catalyzes the conversion of AA to LTA$_4$. 5-LO activity is mostly regulated through its subcellular localization within the cell. 5-LO contains nuclear transport signals at its C- and N-terminal regions and is found in the cytosol or nucleus depending on the cell type [60, 61]. Translocation of 5-LO from the cytoplasm to the nucleus is calcium dependent and is thought to be mediated through N-terminal β-barrel which resembles a C2 domain [62, 63]. Tryptophan residues in C2-like domain are required for binding to phosphatidylcholine in a calcium-dependent manner [63] and is further augmented by AA itself [64]. However, nuclear import may also take place before cell stimulation - as in the case of alveolar macrophages [65] - or may be enhanced by priming of the cell or by cell adhesion as in the case of neutrophils [66]. Interestingly, the nuclear presence of 5-LO in resting neutrophils has been reported to be gender specific and is ERK-dependent in response to androgens [67]. Nuclear import also appears to depend on MAPK pathways other than ERK [68] and has been shown to be facilitated by 5-LO interaction with a coactosin-like protein [69]. Upon stimulation, cytosolic and nuclear 5-LO translocate to the inner and outer nuclear membrane in a calcium-dependent manner, although transport of the enzyme across the perinuclear space has not yet been ruled out [70]. The localization of

5-LO with either the inner or outer nuclear membrane leads to the generation of *HpETE and LTA$_4$* in distinct cellular compartments, which presumably account for the distinct functions of LTB$_4$ in a cell type specific manner [71].

Regulation of LTA$_4$H

LTA$_4$H is a zinc containing metalloenzyme with intrinsic aminopeptidase activity and an exquisite stereospecificity to substrates containing 5,6-trans-epoxide, like 5-HpETE [72]. LTA$_4$H is expressed in wide variety of tissue, even those that lack significant 5-LO activity e.g. endothelial cells, erythrocytes, and lymphocytes. LTA$_4$ is, hence, thought to be exported from a producer cell to a LTA$_4$H-containing recipient cell where it can convert LTA$_4$ to LTB$_4$ [73]. This example of transcellular biosynthesis of LTB$_4$ provides a key regulatory mechanism for LTB$_4$ biosynthesis and tissue availability. The other key regulation mediated by LTA$_4$H is its suicide inactivation by its own substrate LTA$_4$. LTA$_4$ covalently modifies LTA$_4$H to irreversibly inactivate the enzyme, thereby shutting down LTB$_4$ biosynthesis. The final regulatory step mediating LTA$_4$H function is the subcellular localization of the enzyme [74]. Unlike 5-LO, LTA$_4$H has been purified as a soluble protein and its activity is not expected to depend on membrane association. However, LTA$_4$H localizes in the nucleus in a cell-type dependent manner. For example, LTA$_4$H resides in the nucleus of alveolar macrophages and rat basophilic leukemia cells, but is predominantly cytoplasmic in neutrophils [75]. Interestingly, neutrophils show higher vesicular, punctate cytoplasmic localization of LTA$_4$H upon adhesion compared to cells in suspension [75]. This observation will become important when we discuss vesicular packaging of LTB$_4$.

Distinct roles of intracellular and extracellular LTB$_4$

Nuclear LTB$_4$—The direct action of LTB$_4$ in the nucleus occurs in conjunction with the nuclear receptor PPARα [76]. *In silico* docking studies suggests that LTB$_4$ and other AA metabolites, such as 5-HpETE and LTA$_4$, can directly bind to PPARα and can mediate PPARα action on the transcription of target genes [77]. More importantly, binding of LTB$_4$ to PPARα has been shown to represent an alternate level of control of the downstream effects of LTB$_4$ during inflammatory responses. Indeed, β- and ω-fatty acid oxidation mediated by LTB$_4$-induced PPARα activation leads to the breakdown of LTB$_4$ and thereby act as a potential feedback mechanism during inflammation resolution [78].

Cellular transport of LTB$_4$ and formation of chemotactic gradients in tissues

Once synthesized in the cytosol, LTB$_4$ is released into the extracellular environment, where it acts in an autocrine and paracrine fashion by binding to surface receptors (see below). The mechanism by which this occurs is not obvious given the low solubility of eicosanoids and the requirement for a specific transport mechanism. It is known that LTB$_4$ is rapidly released following cellular activation [79] and that free cytosolic intracellular LTB$_4$ does not accumulate in cells [80, 81]. Moreover, the release of LTB$_4$ is a saturable process that is dependent on temperature and involves a carrier [82]. MRP4, a member of the ATP binding cassette subfamily C, has been proposed as a candidate carrier as membrane vesicles containing ABCC4 (MRP4) are capable of transporting LTB$_4$ in the presence of glutathione [83]. Neutrophils treated with an inhibitor of MRP4 show reduced chemotaxis and LTB$_4$

release in response to IL8. However, inhibition of MRP4 itself stimulated the release of LTB$_4$, suggesting that MRP4 may only be involved in the ligand induced synthesis or transport of factors required for LTB$_4$ synthesis [84]. Nevertheless, a transporter-mediated process would not explain how a hydrophobic molecule such as LTB$_4$ can maintain its stability in circulation and form stable gradients in tissue, which are required for immune cell chemotaxis. Stable gradients of biomolecules have been proposed to be maintained by reaction-diffusion systems, where the buildup of the molecule is prevented by degradation/ inactivation by ectoenzymes [9]. Maintenance of LTB$_4$ gradients through specific inactivation by β-oxidation although possible, does not seem to be efficient given the very short half-life of LTB$_4$ in vivo (less than half minute), which is very much like the β-oxidation resistant LTB$_4$ analogs [85]. In fact, when compared to primary chemoattractants like formyl peptides, the release and the subsequent diffusion of AA, the synthetic precursor for LTB$_4$, is extremely transient and does not create effective gradients [86]. While the stability of LTB$_4$ may be enhanced in systemic circulation by binding to serum albumin [87], this would not explain its stability in interstitial spaces. Alternatively, stable and long acting LTB$_4$ gradients could be maintained by facilitated diffusion through transcytosis, vesicle-mediated transport or packaging in extracellular vesicles such as exosomes [9]. Vesicular packaging represents an especially efficient process for dissemination of hydrophobic molecules. For example, lipid adduct morphogens in *Drosophila* such as Wnt are known to be released from cells in extracellular vesicles called argosomes [88]. Even non-lipid chemoattractants such as chemokines have been shown to be associated with extracellular vesicles [88]. In the social amoeba *Dictyostelium* cAMP, the primary chemoattractant that governs its development, has been proposed to be released through vesicular packaging [11].

Vesicular packaging of LTB$_4$ - implication in chemotaxis and signal relay

Extracellular vesicles (EV) are small membrane bound organelles that are released from cells to the extracellular milieu. EVs derived from the plasma membrane (PM) are typically larger (~ 200 nm- 2 μm) and are called secretory vesicles, whereas smaller vesicles (~ 50-150 nm) derived intracellularly from multivesicular bodies are called exosomes. These EVs, initially thought to be cellular trash have been shown to mediate varied immune processes [89]. For example, exosomes from bronchoalveolar lavage fluid of patients with sarcoidosis contain FLAP, LTA$_4$H and 5-LO and increase leukotriene production by bronchial epithelial cells [90]. Similarly, exosomes isolated from macrophages and dendritic cells contain the three LTB$_4$ synthesizing enzymes [91] and are thus capable of LTB$_4$ synthesis. Active cPLA$_2$ and other eicosanoids are also released from exosomes derived from monocytic RBL-2H3 cells [92]. Recently, vesicular packaging of LTB$_4$ was directly demonstrated in migrating neutrophils. Remarkably, the vesicular redistribution of 5-LO from the nuclear envelope into MVBs was readily observed in these cells upon fMLP-treatment. Furthermore, the secreted exosomes released LTB$_4$ in a time-dependent manner, which was poised to activate neutrophils through autocrine or paracrine mechanisms. Further, the inhibition of exosome production lead to a decrease in exosomal LTB$_4$ release and a loss in directed migration [14]. Together, these reports suggest that LTB$_4$ and its synthesizing enzymes are packaged in exosomes, which upon secretion causes further release of LTB$_4$ in a time-dependent manner to form a secondary chemoattractant gradient.

This secondary gradient of LTB_4 becomes the 'bread-crumbs' for recruiting and guiding incoming follower cells, as the primary chemoattractant gradient becomes too shallow to be effectively perceived. Exosomes may therefore amplify primary chemoattractant gradients by relaying their signals through LTB_4. Our current understanding on the production and exosomal packaging of LTB_4 in neutrophils is summarized in the Figure 1.

THE BIOLOGY OF LTB_4 RECEPTORS

LTB_4 receptor properties

LTB_4 can bind and activate two distinct GPCRs - BLT1 and BLT2. BLT1 was cloned from the leukemic cell line HL-60 upon differentiation with retinoic acid [93]. It has a high affinity for LTB_4, with a Kd of ~ 0.1-2 nM depending on the cell type [93, 94]. BLT2 was identified using the BLT1 sequence as a template and binds LTB_4 with lower affinity (~20 nM) [95]. LTB_4 binding to BLT1 requires the specific carbon backbone arrangement and the hydroxyl groups of LTB_4. Importantly, LTB_4-BLT1 interaction cannot be competed out by the presence of related lipids such as 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) or 15(S)-HETE. However, the binding of LTB_4 to BLT2 can be competed out by 12(S)-HETE, 12(S)-HPETE or 15(S)-HETE [96]. In fact, 12(S)-hydroxyheptadecatrienoic acid (12-HHT), which is abundantly produced by platelets during blood coagulation, competes out LTB_4 at a much lower concentration and has been described as the naturally occurring high-affinity *in vivo* ligand for BLT2 [94, 97]. Interestingly, the BLT1 promoter is also a part of the BLT2 open reading frame (ORF), which is upstream of BLT1 in the genome, raising the possibility of their co-regulation under certain conditions [95]. BLT1 and BLT2 belong to the family A of GPCRs consisting of Rhodopsin/β2-adrenergic-like receptors [98]. They are further subcategorized under the "non-chemokine chemoattractant receptors" group that includes formyl peptide receptors (FPR1 and FPR2), platelet activating factor receptor (PAFR), and complement-3/-5a receptors (C3aR and C5aR) [6].

Cellular distribution of LTB_4 receptors

The expression of BLT1 and BLT2 has been investigated in a variety of tissues and cell types under a plethora of conditions. Generally, BLT2 is more widely expressed in tissues such as heart, lung, liver, kidney, pancreas, prostate gland, and ovary than is BLT1 [93–95, 98]. Innate immune cells such as neutrophils, monocytes, macrophages, eosinophils, basophils, mast cell and dendritic cells represent the most prominent group of cells that express BLT1. Interestingly, BLT2 is expressed in most of the above-mentioned innate immune cells except for basophils and macrophages. The ability of lymphocytes - T and B cells - as well as endothelial cells to specifically express BLT1, but not BLT2, is also intriguing [98].

G protein specificity of BLTs

BLT1 and BLT2 are characterized by the presence of a DRY motif within their second intracellular domain, which is known to interact with the $G\alpha i$ subunit of the heterotrimeric G protein complex [6]. Initial studies showed that BLT1 can couple to both $G\alpha i$ and Gq subunits [93]. However, it was subsequently established that the LTB_4-dependent BLT1 receptor activation is sensitive to pertussis toxin and therefore a $G\alpha i$-mediated event [93,

99–101]. Interestingly, and unlike BLT1, BLT2 can associate with different G proteins, such as Gα14 and to a lesser extent with Gαq and Gα11 [97].

The role of helix8 and GRKs in BLT trafficking

The binding of a ligand to its cognate GPCR leads to a conformational change in the receptor resulting in loading of GTP to the Gα subunit of the heterotrimeric G protein complex [101]. This stage represents the sensitized state of the GPCR. Once the receptor is activated, the GTP-loaded G protein is released from the receptor and the Gα and βγ subunits then dissociate to regulate downstream signaling pathways [98, 102]. The G protein-dissociated receptors then become prone to phosphorylation of Ser/Thr residues in their intracellular C-terminal tail by GPCR regulated kinases (GRKs), protein kinase C (PKC) or cAMP-dependent protein kinases (PKA). This step marks the desensitization of the receptor. Once phosphorylated, the tail of the GPCR becomes susceptible to loading of arrestin molecules, which then recruit the adapter AP2 molecule to help dock clathrin, a mediator of receptor endocytosis [101, 102]. Among the different intracellular domains, the C-terminal tail from the seventh transmembrane domain harbors the intracellular helix 8, which is critical for a subset of GPCRs responses, including desensitization and internalization [94]. Surprisingly, BLTs lack the conserved cysteine residue within helix 8, which upon palmitoylation anchors the helix 8 of Class A GPCRs to the PM. Instead, based on modeling studies, hydrophobic di-leucine residues (Leu304-Leu305) within helix 8 are predicted to anchor helix 8 of BLT1 to the PM [101]. However, conversion of the di-leucine residues to alanine residues (LL/AA) had little impact on the ability of the mutant to localize to the PM. Yet, upon LTB_4 stimulation, while the LL/AA mutant exhibits resistance to internalization when expressed in COS-7 cells [103], the same mutant shows higher internalization compared to wild-type (WT) receptor in CHO cells [104]. Interestingly, the ability of the LL/AA mutant to bind LTB_4 and subsequently induce inositol phosphate (IP) accumulation is better than that of WT BLT1 in both of these studies. In terms of trafficking, unlike WT BLT1, the LL/AA mutant strongly co-localizes with clathrin upon stimulation with LTB_4 [104]. BLT1 internalization is dependent on dynamin activity, but surprisingly independent of β-arrestin-2 that is generally involved in clathrin-mediated endocytosis of GPCRs [105].

The major drivers of BLT1 internalization are GRK2, GRK5 and GRK6 [105]. In cells that express low endogenous GRK2 amounts, such as HEK293 and COS-7, GRK6 regulates BLT1 endocytosis by phosphorylating Thr308 residue in helix 8 [105, 106]. In contrast, in cells that express high amounts of GRK2, BLT1 endocytosis is dependent on the interaction of GRK2 with the C-terminal tail of BLT1 upon LTB_4 stimulation [105]. Therefore, it is possible that multiple GRKs can interact with BLT1 simultaneously to affect its internalization in a dynamin-dependent manner. With regards to BLT2, helix 8 is required for efficient transport of BLT2 from the ER to the PM. Also, addition of agonists for BLT2, such as 12-HHT, promote the re-localization of the LL/AA mutant of BLT2 from the ER to the PM, exhibiting a chaperon-like effect of the ligand [107]. The trafficking of WT and LL/AA BLT1 and BLT2 mutants remains to be studied during neutrophil migration, where the LTB_4-BLT1 signaling axis is required for efficient chemotaxis *in vitro* and *in vivo* [12, 13].

Regulation of signaling pathways upon BLT activation

The activation of BLT1 and BLT2 regulates a plethora of downstream signaling events that impact on a wide array of cellular functions. However, in the context of this review we will restrict our discussion to the specific signaling cascades that contribute to BLT-induced leukocyte migration. BLT1 is known to mediate migration of several cell types *in vitro* and *in vivo*, including vascular smooth muscle cells [108], neutrophils [109], eosinophils [110], macrophages [111], mast cells [112], CD8+ T cells [113], Th17 cells [114], Treg cells [115] and γδ T cells [116]. On the other hand, BLT2 has been shown to regulate migration only in neutrophils [109], mast cells [112] and dendritic cells [117]. It is also important to point out that LTB$_4$-BLT1/BLT2-mediated signaling events in leukocytes have not been completely deciphered, compared to the events mediated by fMLP or IL8.

Upstream phosphatidylinositol signaling

In the context of leukocyte migration, GPCRs activate a variety of upstream signaling events including phospholipase β (PLCβ), Ras and other small GTPases that dictate polarity and directional migration [3]. Once dissociated from the G protein heterotrimeric complex, Gβγ subunits activate PLCβ, which in turn catalyzes the breakdown of phosphotidylinositol(4,5)bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). Both DAG and IP$_3$ then act as second messengers to transduce downstream signals [102]. In neutrophils, activation of Ras is dependent on PLCβ$_2$ and PLCβ$_3$, which activate a Ras guanine exchange factor (RasGRP4) to promote Ras activation [118]. Chemoattractants such as fMLP and LTB$_4$ induce the production of phosphotidylinositol(3,4,5)trisphosphate (PIP$_3$) in neutrophils [119]. This occurs following Ras binding and activation of the Class IB phosphatidylinositol 3-kinase (PI3K), p110γ [120]. Indeed, PI3Kγ-dependent production of PIP$_3$ contributes to neutrophil chemokinesis and chemotaxis [120–122].

Small GTPase activation and actin regulation

The dynamic actin polymerization present at the leading edge of migrating leukocytes is dependent on the presence of a branched actin network that is regulated by the Arp2/3 complex [3] [123]. The activation of the Arp2/3 complex is in turn dependent on Rac driven SCAR/WAVE and Cdc42 driven WASP activation [3]. Filamentous actin (F-actin) production at the leading edge of neutrophils in response to stimulation with chemoattractant is in part dependent on the activity of PI3K/AKT/PKC [124]. PIP$_3$ binds and activates the Rac GEF DOCK2, thereby activating Rac and in turn F-actin assembly, neutrophil polarization and chemotaxis [125]. Importantly, activated Rac feeds back to further increase the production of PIP$_3$ during neutrophil chemotaxis [126]. Apart from Rac, PIP$_3$ is required for the activation of another small GTPase Cdc42 [126] and LTB$_4$ is known to activate both these small GTPases during dendritic cell migration [127]. Although Cdc42 does promote actin nucleation through WASP and Arp2/3 complex, Cdc42 is perhaps more important for the maintenance of polarity and suppression of lateral protrusions during neutrophil chemotaxis [126, 128, 129]. Overall, specific signaling cascades activated downstream of GPCR stimulation are dedicated to promote actin dynamics at the front to drive pseudopod extension and thereby leukocyte migration. However, it remains unclear as to why

neutrophils require the LTB_4-BLT1 signaling axis to efficiently enhance cAMP production, F-actin assembly and to maintain neutrophil polarity in response to a primary chemoattractant such as fMLP [12].

Molecular players in back retraction

For efficient chemotaxis, the signals that regulate back retraction of a migrating leukocyte are just as important as the events dedicated to regulate front protrusions. Just as PIP_3, Rac and Cdc42 regulate actin polymerization at the leading edge, PIP_2, Rho, Rho-associated kinase (ROCK), Myosin II light chain kinase (MLCK) and Myosin II dictate posterior dynamics during neutrophil chemotaxis [3, 130]. Importantly, in response to fMLP much of the active Ras, Rac and Cdc42 preferentially localize to the front while RhoA is restricted to the center and back in chemotaxing neutrophils [131]. The restricted activity of RhoA is important, as dominant-positive RhoA mutants inhibit neutrophil polarity and dominant-negative or inhibition of Rho leads to the formation of multiple front protrusions accompanied with elevated Rac2 activity and delayed back retraction [132]. The interplay of specific molecules is required for the spatio-temporal regulation of Myosin II light chain phosphorylation, which in turn regulates back retraction. For ROCK-dependent MLCK to phosphorylate myosin light chain, calcium from intracellular stores released upon IP_3 production in response to GPCR stimulation is required [133]. Another signaling cascade that is important for Myosin II-mediated back retraction is the mammalian Target of Rapamycin Complex 2 (mTORC2)-dependent cAMP generation, which feeds into the RhoA pathway [134, 135]. In the context of neutrophil signal-relay, LTB_4 acts in a paracrine as well as autocrine manner to facilitate polarity and persistence of chemotactic neutrophils to a primary attractant such as fMLP. This is explained in part by the lack of sustained phosphorylated myosin light chain when the LTB_4-BLT1 axis is inhibited in response to fMLP, which most-likely impacts back retraction [12]. However, the molecular dependence of FPR1 on BLT1-mediated myosin light chain phosphorylation for back retraction is an open area of research.

LTB_4 and leukocyte adhesion

Adhesion to and detachment from the substrate have a central role in the migration of a cell. In the case of neutrophils as well as other leukocytes, the ability to maintain low-adhesion and detachment occurs in the back, and, not surprisingly, several adhesion molecules such as P-Selectin ligand 1 (PSGL1), CD11a and intercellular adhesion molecule 1 (ICAM1) are found in the back of migrating neutrophils [130, 136]. Integrins are a class of adhesion molecules expressed by mammalian cells, including leukocytes, that are rapidly activated by chemoattractants to signal 'inside-out' and thereby mediate adhesion and leukocyte migration [137]. This is in contrast to observed endothelial and fibroblast cell migration, which require 'outside-in' integrin signals accompanied by focal adhesion kinase activation and downstream signaling to drive motility [138]. The ability of LTB_4 and other chemoattractants to induce adhesion of neutrophils to endothelial cells depends on β2 integrins – CD11a, CD11b, CD11c - and the common β chain CD18 [139, 140]. Importantly, perturbation of integrin molecules, directly or indirectly, impacts leukocyte migration behavior [141, 142]. Although chemoattractants in general induce β2-integrins clustering, mobility and 'inside-out' activation in neutrophils, LTB_4 induces a modest yet

significant increase in integrin affinity when compared to fMLP [143]. Also, unlike fMLP, LTB_4 fails to promote the translocation of β1-integrins to the PM and migration through fibrin [144]. Interestingly, while LTB_4 and other chemoattractants promote β2-integrin expression on the PM, the adhesion molecule $gp100^{MEL-14}$ is actively shed from the surface following stimulation [145].

The small GTPase Rap1 contributes significantly to adhesion, polarity and migration of leukocytes including neutrophils. Rap1 is activated downstream of Ras upon GPCR stimulation in neutrophils [118] and activation of β1-, β2- and β3-integrins depends on the activity of Rap1, which in turn is dependent on the exchange factor CalDAG-GEFI. In the absence of CalDAG-GEFI, impaired actin arrangement at the leading edge of neutrophils is accompanied by defective chemotaxis towards LTB_4 [146]. In fact, the constitutively active Rap1aQ63E mutant or overexpression of the Rap1 effector Radil, induces greater adhesion and reduced back retraction in neutrophil-like cells [147]. Although, integrins are not necessary for neutrophil chemotaxis in tissues, it is important to point out that cells lacking Talin, a regulator of integrin activation, of BLT1 have strikingly similar phenotypes in terms of their inability to penetrate collagen-free zone upon injury *in vivo* [13], suggesting that LTB_4 signaling is required for integrin activation. The signaling events driving BLT1 trafficking, neutrophil polarity and adhesion in response to LTB_4 stimulation is pictorially depicted in Figure 2.

LTB_4 and MAPK activation

The other signal transduction pathway downstream of GPCR activation is the mitogen associated protein kinases (MAPKs). LTB_4 can activate MAPKs – ERK, p38MAPK and JNK - in multiple cell types such as fibroblasts [148], neutrophils [149] [150] and pancreatic cancer cells [151]. In neutrophils, the activation of ERK and p38MAPK, but not JNK, are dependent on PI3K activity upon fMLP stimulation [152]. Although evidence for the role of ERK during chemotaxis is yet to be clearly established, it is required for the survival and anti-apoptotic effects of LTB_4 on neutrophils [150]. Similarly, while LTB_4-induced JNK activation has been shown to regulate chemokine production in monocytes, the role of JNK activation during neutrophil chemotaxis is yet to be clearly established [153]. On the other hand, p38MAPK activation is required for efficient chemotaxis to a number of chemoattractants including LTB_4, fMLP and C5a [122]. Although competition of p38MAPK with GRK2 to bind and phosphorylate FPR1 has been proposed [154], the exact mechanism by which activated p38MAPK impacts neutrophil chemotaxis to different chemoattractants remains unclear.

Contrasting the actions of LTB_4 to related lipid mediators

Other LTB_4-related ecosianoids that bind to specific GPCRs have very different effects from those of LTB_4 on target cells and thereby immune responses. For example, intraperitoneal injection of LTC_4, unlike LTB_4, fails to induce neutrophil recruitment in mice [155]. While LTB_4 is produced by fMLP-stimulated neutrophils and is essential for the persistence of directionality during chemotaxis [12], the addition of a BLT2 agonist, 12-HETE, suppresses fMLP-induced calcium flux and migration of neutrophils [156]. Also, Lipoxin A_4 (LXA_4) that binds to LXA_4R or FPR2, a receptor highly homologous to FPR1, inhibits fMLP-

induced CD11b up-regulation and aggregation of neutrophils and thus exhibits anti-inflammatory properties [157]. In fact, pre-treatment of neutrophils with LXA_4 or Lipoxin B_4 (LXB_4), a structurally distinct member of the lipoxin family that signals differently from that of LXA_4, inhibits responses such as calcium flux and chemotaxis to LTB_4 as well as to fMLP [158]. Interestingly, LXA_4 treatment blocks the binding of Jurkat cells to endothelial cells in response to the cytokines IL1β and TNFα and inhibits E-Selectins, ICAM1 and vascular cell adhesion molecule 1, VCAM1 [159]. Similarly, LXB_4 inhibits degranulation of mast cells and eosinophils, reduces eosinophil chemotaxis and represses type 2 cytokine receptors in a murine airway allergic inflammation model [160]. Overall, LTB_4 and structurally related ligands bind to distinct GPCRs and while some of them appear to promote leukocyte function during inflammation, others actively suppress activation and effector functions of leukocytes.

THE PHYSIOLOGICAL ACTIONS OF THE LTB$_4$-BLT1 AXIS

Competing chemoattractant gradients in leukocyte migration

The release of cellular LTB_4 is usually a consequence of tissue damage, infection or other insults. LTB_4 is critical to amplify chemotactic responses to a primary chemoattractant [12]. However, immune cells, such as leukocytes, encounter mixed and opposing gradients of chemoattractants *in vivo*. For example, during endotoxemia, a pathophysiological global activation of inflammatory responses due to bacterial endotoxins, leukocytes encounter both bacterial-derived formylated peptides as well as host-derived chemoattractants such as LTB_4 and IL8 [161]. Interestingly, under these conditions, the response of neutrophils to host-derived chemoattractants is reduced, whereas chemotaxis towards bacterial peptides remains unaffected [162]. Hence, a hierarchical preference for certain chemoattractants has been proposed for leukocytes. Indeed, it has been shown *in vitro* that leukocytes respond preferentially to end-target chemoattractants, such as bacterial peptides, over other host-derived factors or intermediate chemoattractants, such as LTB_4 [163]. This ability to distinguish exogenous cues enables leukocytes to differentiate immediate threats over host-derived endogenous chemoattractant gradients.

A signaling preference in chemoattractant hierarchy

The terms end-target and intermediate chemoattractants are also relevant in terms of the signaling pathways they elicit. Neutrophils predominantly migrate to end target chemoattractants in a p38MAPK-dependent manner, whereas migration to intermediate chemoattractant such as LTB_4 and IL8, is more PI3K/AKT-dependent [8]. The negative bias of neutrophils towards LTB_4 in the presence of end-target chemoattractants is most likely because of the inhibitory effects of p38MAPK on the molecule PTEN as *PTEN$^{-/-}$* neutrophils can be distracted by an intermediate chemoattractant gradient while migrating towards fMLP [164]. Interestingly, cells moving towards a very shallow concentration gradient of fMLP show higher chemotactic response time and speed in the presence of LTB_4, as compared to gradients of only LTB_4 or fMLP [165]. Similarly, monocyte migration to fMLP increases when cells are pretreated with LTB_4, although the reverse, i.e. migration to LTB_4 when pretreated with fMLP, remains unaffected [166]. Moreover, neutrophils treated with LTB_4 receptor antagonists show decreased motility towards fMLP, indicating

that the fMLP-induced release of LTB_4 is important for neutrophil motility towards an end-target chemoattractant [14, 167]. Taken together, these findings would suggest that although the p38MAPK pathway has an overriding effect on the PI3K-AKT pathway, the reverse may not be true and may be dependent on the intensity of its activation. Although leukocyte migration in competing gradient is expected to be frequent, if not common during infection and inflammation, only a handful of studies have addressed such a phenomenon. Establishment of signaling hierarchy in competing gradients requires further investigations involving parameters such as signal strength, time and stochastic variation within cellular population. Single cell measurements of key signaling pathways in a spatio-temporal manner will also help further resolved these issues.

The IL8-LTB_4 power struggles

Migration of cells towards opposing intermediate chemoattractant, *e.g.* LTB_4 versus IL8, seems to be less obvious and assay dependent. In under agarose assays, leukocyte migration is not dependent on either LTB_4 or IL8, but gradient shape and distance between the two sources [168]. Indeed, it has been established that neutrophils migrate toward the more distant attractant source and away from the more proximal one, independently of the chemoattractant species, by integrating signals from both species [169]. Microfluidic chambers that create linear competing gradients of LTB_4 and IL8 show a weak hierarchical preference of LTB_4 over IL8 in directing neutrophil chemotaxis [170]. This observation is in agreement with *in vivo* conditions found in chronic obstructive pulmonary disease patients where LTB_4 levels correlate strongly with neutrophil accumulation compared to IL8 levels [171]. On the other hand, competing gradients created by microfluidic platforms utilizing shear flow, suggest a reversal of the hierarchical order, with IL8 eliciting preferential neutrophil chemotaxis compared to LTB_4 [172]. The discrepancy in these results may be due to the inherent hydrophobicity of LTB_4 and the calculation of apparent diffusivity of LTB_4. Notwithstanding the order of preference, intermediate chemoattractants such as LTB_4 can contribute to the "chemotactic memory" of the cell that promotes sequential chemotaxis from one chemoattractant to another without losing sensitivity of migration. The lack of ordered preference enables a leukocyte that is migrating towards an IL-8 gradient to turn away and chemotax towards LTB_4 irrespective of the concentration gradients of the individual chemoattractant [173]. The mechanisms that control how such disparate molecules elicit similar physiological responses remain unknown.

The LTB_4-BLT axis in infection and inflammation

In a relatively simple model of injecting LTB_4 intra-peritoneally, BLT1 is required for neutrophil, but not macrophage, infiltration. However, unlike injection of LTB_4 alone, intra-peritoneal injection of a more physiological stimulant such as zymosan or thioglycollate leads to recruitment of neutrophils, macrophages and eosinophils in a BLT1-dependent manner, most likely requiring LTB_4-BLT1 signal-relay downstream to primary inflammatory agents [174, 175]. Similarly, LTB_4 production is known to elicit the recruitment of neutrophils and other leukocytes in several mouse disease models including endotoxemia-induced multiple organ injury [176], diabetic retinopathy [177], abdominal aortic aneurysms [178], sterile inflammation in diabetes [179], contact dermatitis [180], cecal ligation and puncture model of sepsis [181], traumatic spinal cord injury model [182], experimental

autoimmune encephalomyelitis [183] and *T. cruzi* infection [184]. However, in some *in vivo* models, cytokine action precedes and induces LTB_4 production and function. For example, in a mouse model of ovalbumin-induced intra-peritoneal recruitment of neutrophils, LTB_4 production is dependent on the secretion of TNFα by $CD4^+$ T cells [185]. In another mouse model, the production of IL1β and TNFα by mast cells as well as LTB_4 production by macrophages dictates the IL1β-induced recruitment of neutrophils to the peritoneum [186]. The LTB_4-BLT1 axis also promotes the migration of smooth muscle cells during atherosclerosis and intimal hyperplasia *in vivo* and the IL1β-induced expression of BLT1 in these cells appears to be NFκB-dependent [187]. *In vivo*, hemolytic events are known to induce LTB_4 production and the recruitment of neutrophils. Indeed, heme alone can act upon resident macrophages and induce LTB_4 production, which in turn can recruit neutrophils to the peritoneal cavity in mice [188]. The LTB_4-BLT1 axis can also function to mediate much more complex inflammatory scenarios. For example, in the murine inflammatory arthritis model deposition of auto-antibody-antigen complex to the joints triggers complement-mediated reaction and the recruitment of neutrophils in a C5aR-dependent manner. The activation of C5aR precedes LTB_4 release by neutrophils to recruit more neutrophils to the joint [189]. On the other hand, FcγR is activated on the recruited neutrophils that signals through the LTB_4-BLT1 axis to produce IL1, which then promotes further neutrophil recruitment in a CXCR1- and CXCR2-dependent manner [190]. Therefore, although multiple cytokines might be essential for initial immune response in variety of different models, they all converge in activating LTB_4-BLT1 mediated signal-relay for efficient leukocyte recruitment.

In several disease models, LTB_4-BLT1 interaction on specific leukocyte cell types contributes to the observed phenotype. In a viral peptide-induced colitis mouse model, BLT1 promotes the recruitment of specific cytotoxic $CD8^+$ T cells [191]. In mouse models of airway hyper-responsiveness, allergen-sensitized and challenged mice display BLT1-dependent recruitment of neutrophils, eosinophils, dendritic cells, $CD4^+$ and $CD8^+$ effector T cells, which together promote T_H2 type responses in the lungs [192–196]. In humans, BLT1 is up-regulated on T cells from lung transplant recipients and in a mouse lung transplantation model, BLT1 mediates $CD8^+$ T cell recruitment and thereby transplant rejection [197]. In another mouse model, the LTB_4-BLT1 axis mediates the activation of autoreactive T cells and effector leukocyte function to cause autoimmune uveitis [198]. LTB_4-BLT1 interactions in general promote inflammation under most circumstances. However, in a mouse model of Lyme arthritis induced by *B. burgdorferi*, the lack of 5-LO resulted in faster joint swelling, a defective clearance of apoptotic neutrophils by macrophages and lesser uptake of opsonized spirochete by neutrophils [199]. Therefore, the LTB_4-BLT1 axis contributes to inflammatory responses in a context-specific manner.

The LTB_4-BLT axis: extravasation versus tissue migration

It is pertinent to differentiate the effects of LTB_4-BLT1-mediated signal-relay on purely chemotactic responses with that on other leukocyte responses in the context of inflammation. In fact, BLT1 is required for efficient leukocyte arrest in response to LTB_4 in the cremaster muscle venule in mice [174]. Therefore, in most, if not all, of the above mentioned mouse infection and inflammation model systems, the LTB_4-BLT1 axis most

likely contributes to inflammation at multiple levels including rolling, arrest/adhesion to the endothelium, extravasation of activated leukocytes from blood vessels and interstitial chemotaxis. However, in the case of a laser induced ear injury model in mice, neutrophils were labeled and injected in the ear prior to laser injury. In this model, where WT and mutant neutrophils are directly compared in the same tissue, long distance neutrophil recruitment and directionality were 5-LO- and BLT1-dependent [13]. The primary attractants are predicted to be FPR2 ligands and necrosis of the first responding neutrophils, which then use the LTB_4-BLT1 axis to relay signals to neighboring neutrophils to be recruited over longer distances. Interestingly, apart from functioning primarily as a chemoattractant, LTB_4 can also induce neutrophils to deliver elastase in a CD11b-dependent manner to endothelial cells expressing junctional adhesion molecule and thereby promote neutrophil's reverse-transmigration [200]. These and other such examples highlight the adaptation of the LTB_4-BLT1 axis for extravasation, tissue migration and reverse migration of leukocytes *in vivo*. It, however, remains to be determined if exosomal packaging of LTB_4 and its synthesizing enzymes is involved in mediating all of the LTB_4-BLT1 signaling events. Further studies in different immune cell types and under multiple inflammatory conditions are required to determine if such packaging in exosomes applies to LTB_4 and other small signaling molecules that direct leukocyte migration.

LTB_4-related eicosanoids and their distinct chemoattractant properties

Unlike 5-LO products, products of 15-LO, such as LXA_4, function distinctly different from LTB_4 under many circumstances. For example, 15-LO is required for efficient epithelial wound healing in the mouse cornea, most likely by inhibiting KC production and promoting timed neutrophil recruitment to the corneal epithelium [201]. Second, in certain sputum samples from chronic bronchitis patients, amounts of 15-LO and 15(S)-HETE inversely correlate with LTB_4 amounts, suggesting an inhibitory nature of one on the other. Third, treatment of neutrophils with 15(S)-HETE inhibits their ability to produce LTB_4 in response to A23187 or fMLP *in vitro* [202]. Therefore, LTB_4 and structurally related eicosanoids can differentially signal to leukocytes to induce distinct and opposing chemotactic responses.

Unlike BLT1, BLT2 has an anti-inflammatory role in mice as *BLT2$^{-/-}$* mice are more sensitive to dextran sodium sulfate-induced colitis. In this case, BLT2 appears to regulate barrier functions of colonic epithelial cells [203]. Similarly, the 12-HHT/BLT2 axis is important for skin wound closure and healing, most probably by inducing keratinocytes to produce TNFα and MMPs during the process [204]. Importantly, the expression of BLT2 is significantly reduced in CD4$^+$ T cells from the bronchiolar lavage in human asthma patients compared to healthy control subjects. Not surprisingly, unlike BLT1, the lack of BLT2 specifically promotes higher IL-13 producing CD4$^+$ T cells and thereby contributes to eosinophilia in the lungs of a airway hyper-responsive mouse model [205].

PERSPECTIVES

Given that most chemoattractant GPCRs are Gαi-coupled and are expected to activate similar pathways, it will be important to understand how similar signaling cascades activated by structurally distinct ligands can bring about different phenotypic responses in the same

target cells. In this review, we largely focused on the role of the LTB$_4$-BLT1 axis in directing leukocyte migration in a variety of physiologically relevant conditions. We emphasized the intricate events leading to LTB$_4$ synthesis and potential transport within exosomes (Fig. 1). Following the production of LTB$_4$, we discussed aspects of BLT1-mediated downstream signaling essential for efficient leukocyte polarization and chemotaxis (Fig. 2). We also discuss the context in which the LTB$_4$-BLT1 axis functions in a complex and competing gradient environment (Fig. 3). However, it remains to be determined how the LTB$_4$-BLT1 axis regulates specific signaling pathways in response to primary chemoattractants during immune cell migration. Dissecting such intricate and complex signaling networks will undoubtedly lead to a clearer understanding of leukocyte function and a better ability to design drugs that have specific impact on a variety of inflammatory diseases.

Overall, there is no doubt about or on the contribution of the LTB$_4$-BLT1 axis as a relay module in the recruitment of several different cell types in a physiological setting (Fig. 3). However, other small molecules, such as 5-oxo-ETE [206], H$_2$O$_2$ [207] and Redox signaling [208], can also potentially function as drivers of signal-relay among leukocytes in the presence or absence of the LTB$_4$-BLT1 axis. Although the packaging/transport of such small molecules needs further characterization, the above examples highlight the distinct advantage of small molecules that are produced rapidly, in robust amounts and propagated using efficient diffusion mechanisms to form spatio-temporal gradients that are required for the long-distance recruitment of leukocytes during inflammation.

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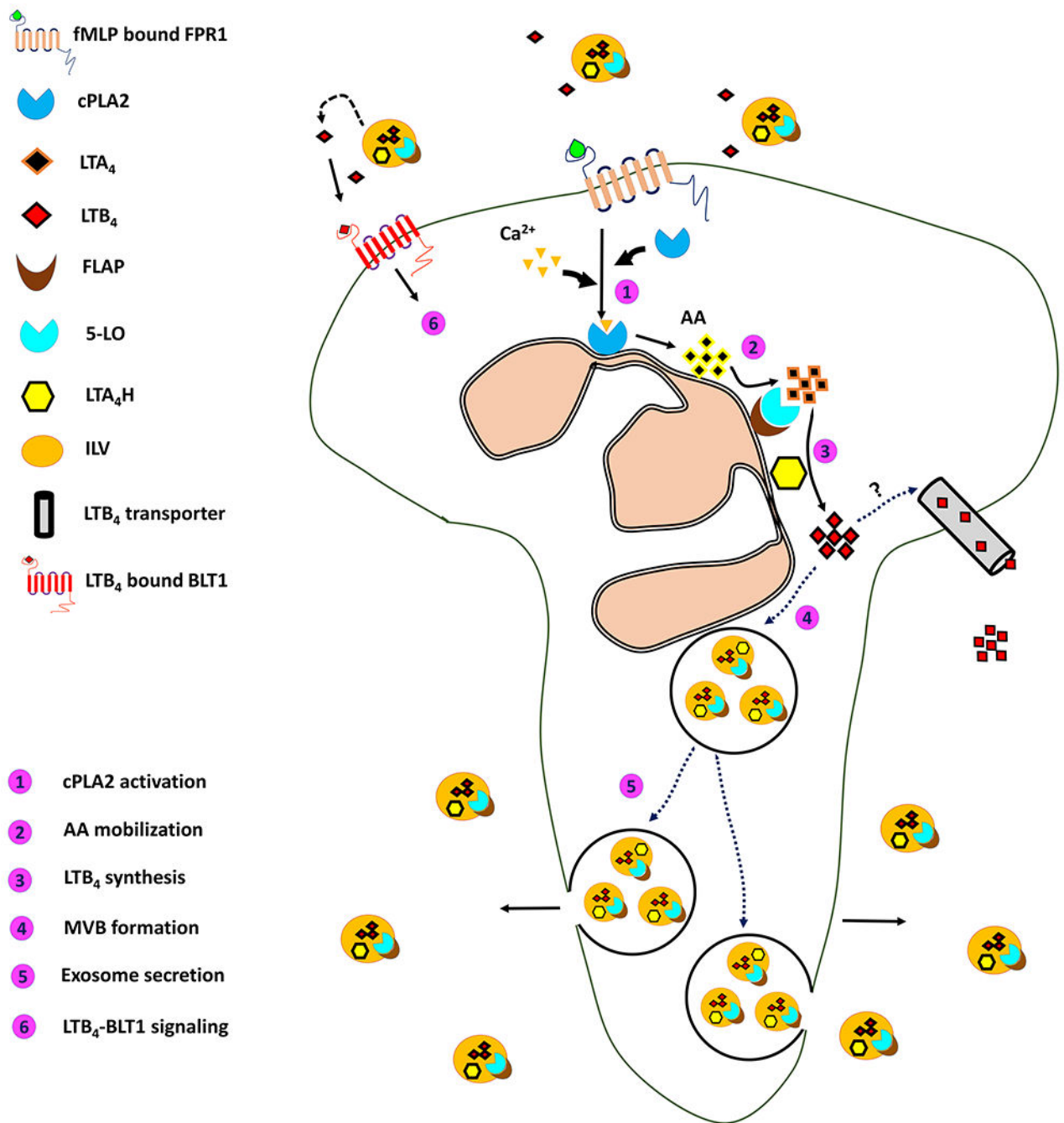
Figure 1. LTB$_4$ synthesis and transport.
A schematic depiction of the various steps that lead to the synthesis of LTB$_4$, it's packaging into MVBs and secretion as exosomes from neutrophils chemotaxing towards a primary chemoattractant.
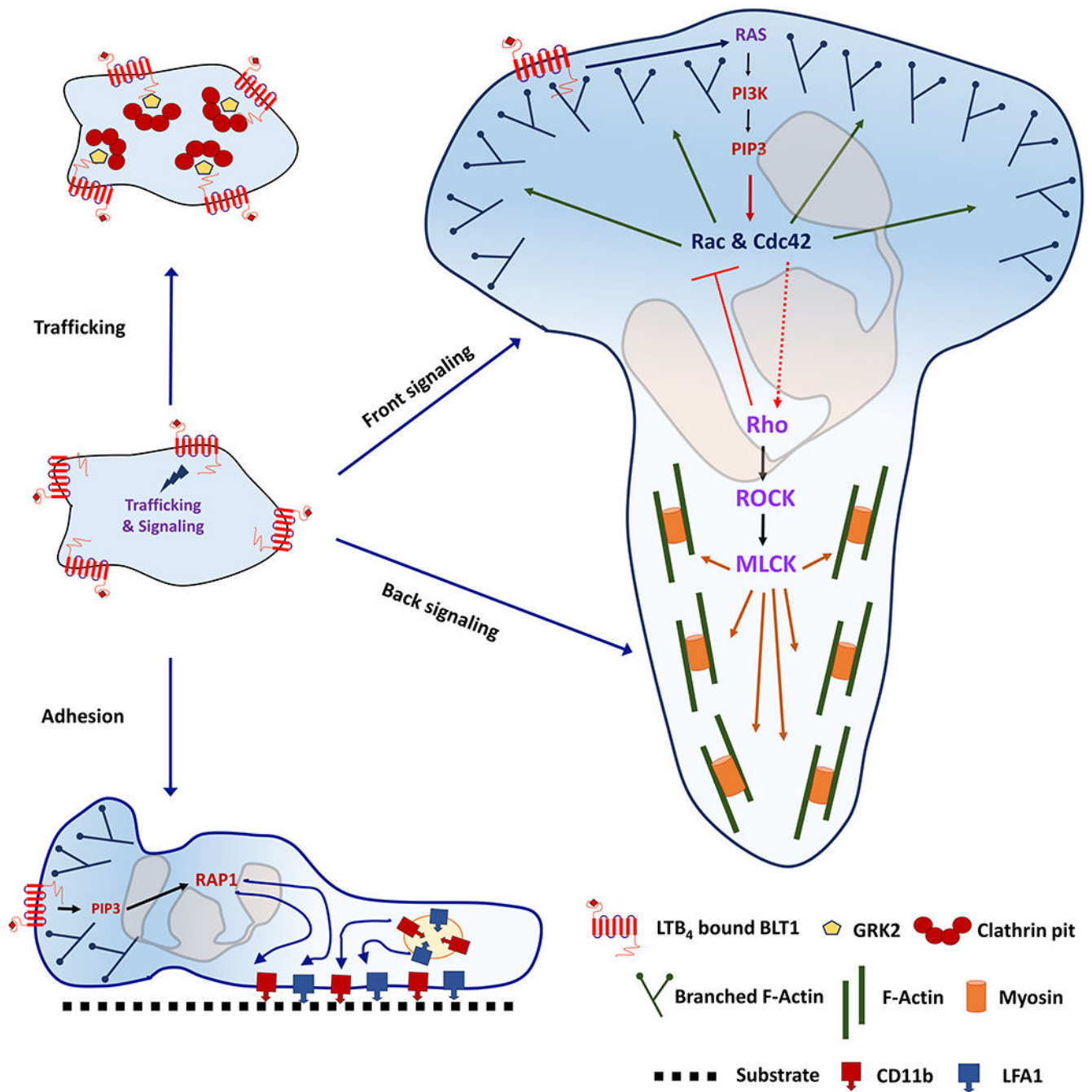
Figure 2. BLT1-mediated signaling in leukocyte.

A schematic representation of the signaling events that occur upon the activation of BLT1 by LTB$_4$. Clathrin-mediated endocytosis of BLT1, front and back signaling as well as inside-out integrin activation in a polarized neutrophil are shown.
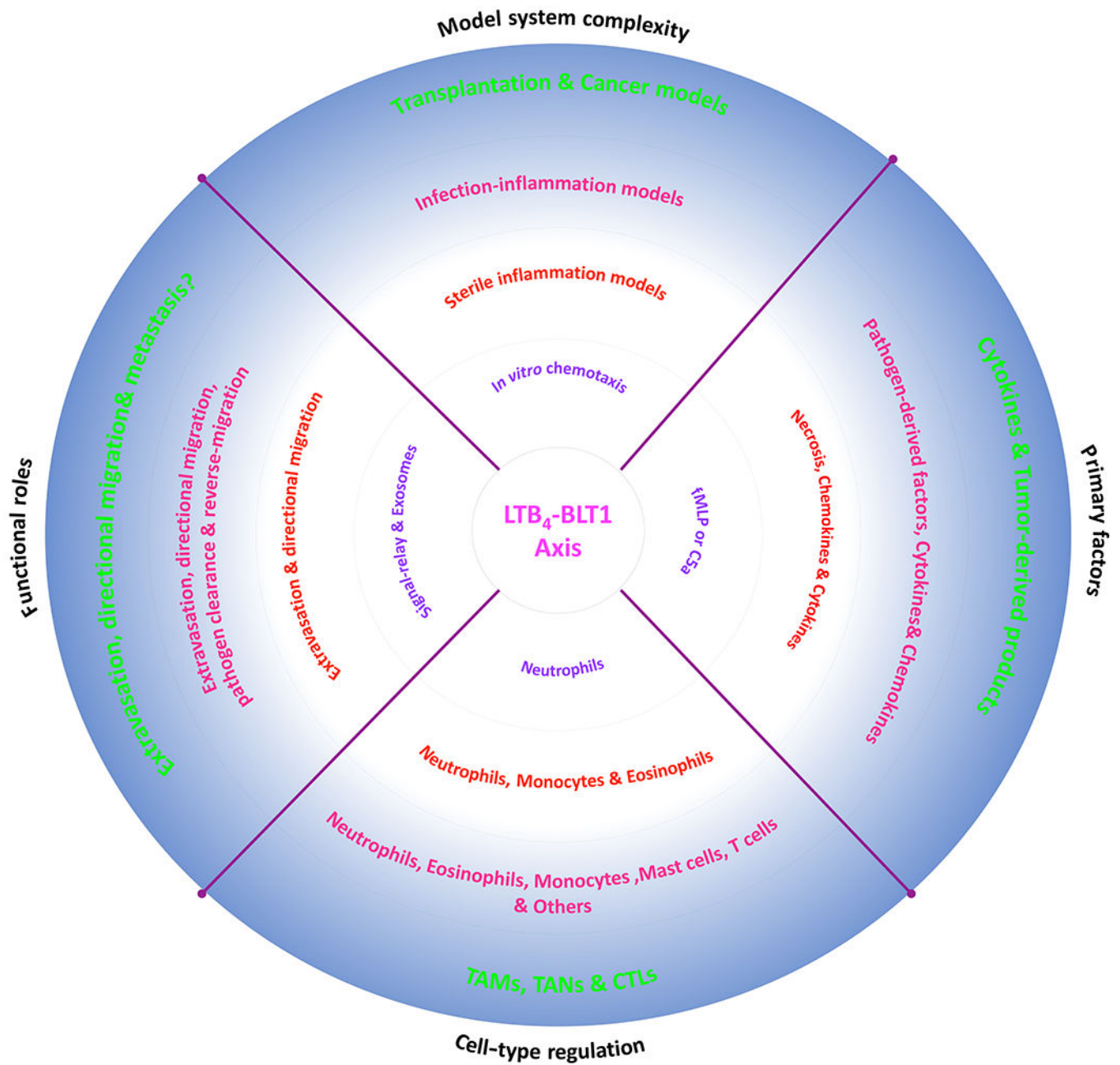
Figure 3. Functional roles of the LTB$_4$-BLT1 axis in diverse cell migration scenarios.
A pie chart depicting the diverse roles of the LTB$_4$-BLT1 axis in distinct situations that involve increased complexities, factors and cell-types.