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LTB_4 Is a Signal-Relay Molecule during Neutrophil Chemotaxis

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SUMMARY

Neutrophil recruitment to inflammation sites purportedly depends on sequential waves of chemoattractants. Current models propose that leukotriene B_4 (LTB_4), a secondary chemoattractant secreted by neutrophils in response to primary chemoattractants such as formyl peptides, is important in initiating the inflammation process. In this study we demonstrate that LTB_4 plays a central role in neutrophil activation and migration to formyl peptides. We show that LTB_4 production dramatically amplifies formyl peptide-mediated neutrophil polarization and chemotaxis by regulating specific signaling pathways acting upstream of actin polymerization and MyoII phosphorylation. Importantly, by analyzing the migration of neutrophils isolated from wild-type mice and mice lacking the formyl peptide receptor 1, we demonstrate that LTB_4 acts as a signal to relay information from cell to cell over long distances. Together, our findings imply that LTB_4 is a signal-relay molecule that exquisitely regulates neutrophil chemotaxis to formyl peptides, which are produced at the core of inflammation sites.

INTRODUCTION

Neutrophils are the most abundant leukocytes in the blood stream and the first cells recruited to an inflammation site, where primary chemoattractants such as formyl peptides released from bacteria or necrotic cells and complement fragments are produced (McDonald et al., 2010). In response to primary chemoattractants, the surrounding tissue as well as resident immune cells, such as macrophages, release secondary chemoattractants (Monteiro et al., 2011; Ribeiro et al., 1997). These proinflammatory mediators activate nearby endothelia and enhance leukocyte extravasation (Soehnlein et al., 2009). After neutrophils have entered the tissue, gradients of secondary chemoattractants guide neutrophils toward the vicinity of the inflammation. Locally, gradients of primary chemoattractants recruit neutrophils to the core of the inflammation (Foxman et al., 1997; Heit et al., 2002). After they have reached the inflammation site, neutrophils in turn secrete secondary chemoattractants and recruit additional leukocytes, which further amplify the inflammation process (Silva, 2010).

It has been proposed that secondary chemoattractants are secreted in sequential waves (McDonald and Kubes, 2010), where leukotriene B_4 (LTB_4) is the first secondary chemoattractant released at an inflammation site (Chou et al., 2010; Kim et al., 2006). LTB_4 is a product of arachidonic acid (AA) metabolism. It is synthesized by the sequential action of 5-lipoxygenase (5-LO) and leukotriene A_4 hydrolase (LTA_4H) (Crooks and Stockley, 1998; Peters-Golden and Henderson, 2007) and mediates its effects by binding to the G protein-coupled receptor BLT-1 (McDonald et al., 1992; Tager and Luster, 2003). LTB_4 is a potent chemoattractant for neutrophils and a key player in the initiation of inflammation (Canetti et al., 2003; Grespan et al., 2008; Ramos et al., 2005). Indeed, Chen et al. demonstrated that the recruitment of neutrophils toward inflammation sites is dependent on 5-LO expression in neutrophils (Chen et al., 2006a).

The current model suggests that LTB_4, as a secondary chemoattractant, is released once neutrophils reach the site of inflammation (McDonald and Kubes, 2010). We hypothesize that LTB_4 is actively secreted by neutrophils as they are migrating toward formyl peptides, therefore acting as a signal-relay molecule. To test this hypothesis, we assessed the role of LTB_4 secretion during primary neutrophil activation and migration in response to formyl peptides. We find that LTB_4 significantly amplifies neutrophil recruitment to primary chemoattractants by selectively modulating signaling cascades involved in cell polarization and by serving as a potent secondary gradient. Thus, LTB_4 acts as a signal-relay molecule for neutrophils migrating toward formyl peptides.

RESULTS

LTB_4 Secretion Does Not Alter fMLP-Induced ERK and PI3K Activation

We show that in response to the formyl peptide fMLP (N-formyl-methionine-leucine-phenylalanine), primary human neutrophils rapidly secrete LTB_4 in a concentration-dependent manner (Figure 1A), as previously established (Dahinden et al., 1988). Because LTB_4 and fMLP both bind to Gαi protein-coupled

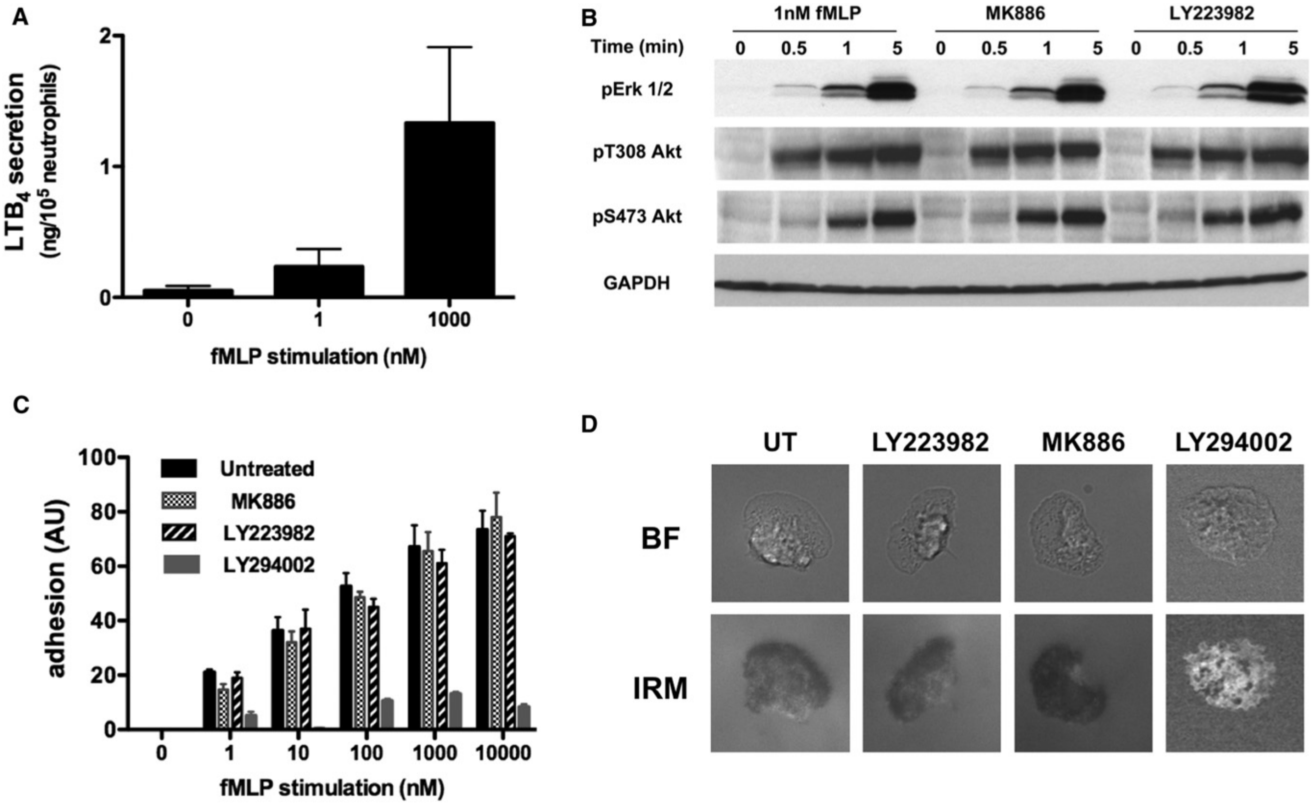

Figure 1. LTB$_4$ Secretion Does Not Alter fMLP-Induced ERK and PI3K Activation

(A) fMLP-induced LTB$_4$ secretion by neutrophils is dose dependent. Primary human neutrophils were stimulated with fMLP for 1 min, and the amount of LTB$_4$ in the supernatant was determined by ELISA. Results represent the average ± SEM of four independent experiments.

(B) LTB$_4$ secretion does not amplify Erk1/2 and Akt phosphorylation upon stimulation with subsaturating doses of fMLP. Primary human neutrophils were stimulated with 1 nM fMLP after pretreatment with either 100 nM MK866, 10 μM LY223982, or DMSO as a control. The western blot for the kinetics of activation is representative of three independent western blot analyses. Also see Figures S1A and S1B.

(C) fMLP-induced LTB$_4$ secretion has no impact on cell adhesion to fibronectin. Primary human neutrophils were treated with either 100 nM MK866, 10 μM LY223982, or 40 μM LY294002, a PI3K inhibitor. Cells were plated on fibronectin-coated plates for 10 min and uniformly stimulated with different concentrations of fMLP. The plates were then shaken, and the number of remaining cells attached to the plates was estimated by crystal violet staining. Results represent the average ± SEM of four independent experiments.

(D) Neutrophil adhesion pattern is not altered upon treatment with LTB$_4$ pathway inhibitors. Neutrophil adhesion to fibronectin-coated plates upon stimulation with 1 nM fMLP was observed by IRM in the presence or absence of drugs as described in (C). The areas of close contact of neutrophils to the substratum appear dark in the IRM image. Representative images are presented.

receptors (BLT-1 and FPR1, respectively) and activate similar cellular pathways (Berger et al., 2002; Cotton and Claing, 2009; Kuniyeda et al., 2007), we set out to determine if signal transduction pathways are amplified by fMLP-induced LTB$_4$ secretion in primary human neutrophils. For this purpose we used two chemical inhibitors: MK886, an inhibitor of 5-LO activity and subsequent LTB$_4$ production (Gillard et al., 1989); and LY223982, a BLT1 receptor antagonist, which blocks LTB$_4$-mediated responses (Jackson et al., 1992).

We first focused our attention on the impact of LTB$_4$ secretion on PI3K activation because previous reports suggested that the PI3K-PTEN axis is specifically involved in neutrophil migration toward LTB$_4$ (Heit et al., 2002, 2008). We observed no significant difference in the fMLP-mediated phosphorylation of Akt on T308 (mediated through PI3K) (Alessi et al., 1997) in the presence of either MK886 or LY223982 compared to untreated cells (Figure 1B and Figure S1A available online). These results are consistent with the fact that LTB$_4$ gives rise to a lower level of Akt phosphorylation compared to fMLP (Figure S1B); any increase in signal mediated by LTB$_4$ would not be significant compared to the response elicited by fMLP alone. Similarly, we found that LTB$_4$ signaling has no effect on the fMLP-mediated phosphorylation of Akt on S473, which is mediated through mTORC2 (Sarbassov et al., 2005), or of Erk1/2 (Figures 1B, S1A, and S1B). Together, these findings establish that LTB$_4$ secretion has no impact on Akt and Erk1/2 activation upon fMLP stimulation.

Because the PI3K pathway has been linked to cell adhesion (Ferreira et al., 2006; Pellegatta et al., 2001; Shimizu and Hunt, 1996), we also tested the impact of secreted LTB$_4$ on neutrophil adhesion in response to fMLP. We found that fMLP stimulation results in a dose-dependent increase in the number of neutrophils adhering to a fibronectin-coated surface (Figure 1C). As previously reported, we also found that PI3K inhibition by LY294002 treatment dramatically reduces the capacity of neutrophil to adhere (Oakes et al., 2009). In contrast, and

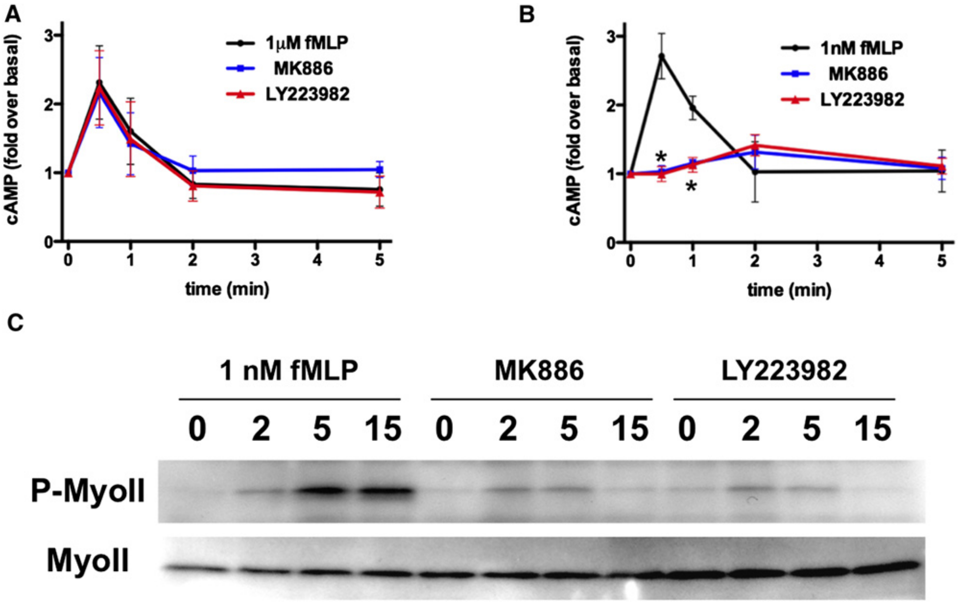

Figure 2. LTB$_4$ Secretion Enhances fMLP-Induced cAMP Production and MyoII Phosphorylation

(A) LTB$_4$ secretion has no impact on intracellular cAMP accumulation in neutrophils stimulated with a saturation dose of fMLP. Primary human neutrophils were treated with 100 nM MK886 or 10 µM LY223982 or DMSO as control and stimulated with 1 µM fMLP. Intracellular cAMP levels were determined by ELISA at the indicated time points. Results represent the average ± SEM of three independent experiments.

(B) LTB$_4$ inhibition reduces cAMP accumulation in neutrophils treated with a subsaturating dose of fMLP. Primary human neutrophils were treated as in (A) and stimulated with 1 nM fMLP. Results represent the average ± SEM of three independent experiments. *$p < 0.05$, ANOVA; Dunnett post hoc test. Also see Figures S2A and S2B.

(C) LTB$_4$ secretion amplifies phosphorylated MyoII levels in response to subsaturating doses of fMLP. Primary human neutrophils were plated on fibronectin-coated plates for 10 min and stimulated uniformly with 1 nM fMLP in the presence or absence of drugs as described in (A). The western blot for the kinetics of activation is representative of three independent western blot analyses. Also see Figure S2C.

consistent with our results of PI3K activation, no alteration in the adhesion capacity of neutrophils was detected in the presence of either MK886 or LY223982 (Figure 1C). Finally, comparison of neutrophil-substrate contact area using interference reflection microscopy (IRM) revealed no significant difference between cell contact areas in response to 1 nM fMLP in the presence of LTB$_4$ pathway inhibitors (Figure 1D). These data confirm that PI3K modulates neutrophil adhesion and is not affected by LTB$_4$ secretion following fMLP addition.

Autocrine and Paracrine LTB$_4$ Secretion Enhances fMLP-Induced Cell Polarization

We recently reported that the fMLP-mediated activation of the adenylyl cyclase 9 (AC9) and the subsequent accumulation of intracellular cAMP are important for neutrophil polarization and back retraction (Liu et al., 2010). We, therefore, set out to determine whether fMLP-induced LTB$_4$ secretion alters intracellular cAMP dynamics at subsaturating and saturating doses of fMLP (FPR1 K_D = 1 nM) (Migeotte et al., 2006). We found that LTB$_4$ pathway inhibitors do not impact the fMLP-mediated cAMP accumulation when fMLP is presented under saturating conditions (1 µM) (Figure 2A). In sharp contrast, both MK866 and LY223982 dose-dependently inhibited the ability of fMLP to induce cAMP production under subsaturation conditions (1 nM) (Figures 2B, S2A, and S2B). These findings establish that LTB$_4$ secretion is required to elicit intracellular cAMP accumulation following stimulation with 1 nM fMLP. Because intracellular cAMP accumulation regulates uropod dynamics via a PKA/MyoII axis (Liu et al., 2010), we next measured the effect of fMLP-induced LTB$_4$ secretion on the extent of myosin light-chain MyoII phosphorylation in neutrophils stimulated with 1 nM of fMLP. In accordance with our cAMP measurements, we found that the levels of fMLP-induced MyoII phosphorylation are significantly reduced in the presence of LTB$_4$ pathway inhibitors (Figures 2C and S2C). These data suggest that fMLP-induced LTB$_4$ secretion affects uropod dynamics during chemotaxis.

We next determined the role of fMLP-induced LTB$_4$ secretion in fMLP-mediated actin polymerization and cell polarity. In response to a uniform stimulation of chemoattractant, neutrophils first accumulate cortical F-actin evenly around their periphery in a so-called cringe response; they then polarize and acquire a network of branched F-actin at their leading edge (Figure 3A) (Orelio and Kuijpers, 2009). When stimulated with 1 µM fMLP, the amount of F-actin in neutrophils doubles within 20 s and remains high up to 5 min (Figure 3B). Under these conditions, 83% of neutrophils accumulate cortical F-actin after 30 s, and 85% of neutrophils are polarized after 2 min (Figure 3D). Pretreating neutrophils with LTB$_4$ pathway inhibitors has no effect on this outcome (Figures 3B and 3D). These results illustrate that the drugs have no toxic effect on the capacity of cells to polymerize actin and that LTB$_4$ secretion has no impact on F-actin dynamics and cell polarization following saturating stimulations of fMLP.

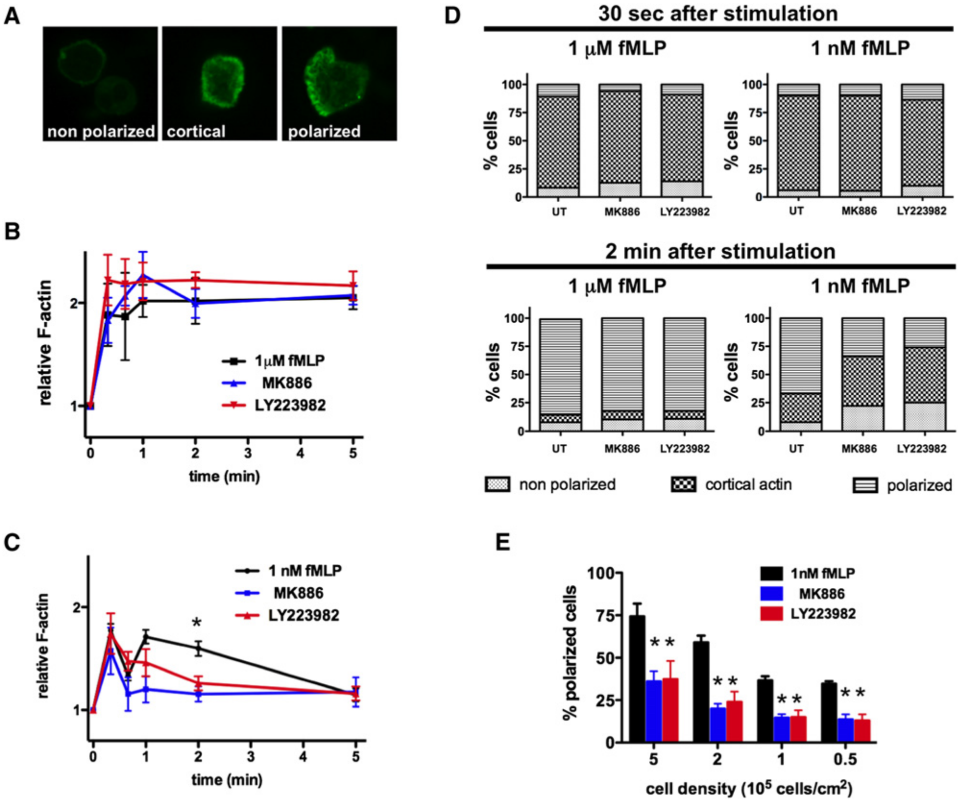

Figure 3. Autocrine and Paracrine LTB$_4$ Secretion Enhances fMLP-Induced Cell Polarization

(A) Different stages of neutrophil polarization can be observed in response to fMLP stimulation. Primary human neutrophils were plated on gelatin-coated plates. Cells were stimulated, fixed, and F-actin was stained with FITC-phalloidin. Representative images are presented.

(B) LTB$_4$ secretion has no impact on neutrophil response to a saturating dose of fMLP. Primary human neutrophils were treated with 100 nM MK886 or 10 μM LY223982 or DMSO as control, stimulated with 1 μM fMLP, fixed, and the F-actin network was stained with FITC-phalloidin. The kinetics of the average fluorescence was determined by FACS analysis. Results represent the average ± SEM of three independent experiments.

(C) Neutrophil treatment with LTB$_4$ inhibitors reduces neutrophil polarization in response to subsaturating doses of fMLP. Primary human neutrophils were treated as in (B), stimulated with 1 nM fMLP, and F-actin levels were determined by FACS, after staining with FITC-phalloidin. Results represent the average ± SEM of three independent experiments. $^*p < 0.005$, ANOVA; Dunnett post hoc test.

(D) LTB$_4$ amplifies neutrophil polarization after 2 min of fMLP stimulation. Primary human neutrophils were treated as in (B), plated on gelatin-coated plates, stimulated with fMLP, and fixed at different time points. Cells were stained with F-actin and counted into three categories (unpolarized, accumulated cortical F-actin, polarized). Results represent the average of four independent experiments.

(E) LTB$_4$ amplifies neutrophil polarization in an autocrine and paracrine manner. Primary human neutrophils were treated as in (B), plated on gelatin-coated plates at different cell densities for 10 min. After 2 min stimulation with 1 nM fMLP, cells were fixed, and the number of polarized cells was counted. Results represent the average ± SEM of three independent experiments. $^*p < 0.05$, ANOVA; Dunnett post hoc test.

See also Figure S3.

When neutrophils are stimulated with the subsaturating dose of 1nM fMLP, the F-actin accumulation follows a biphasic profile with peaks at 20 s and 1 min after stimulation (Figure 3C). The first peak of F-actin correlates in time with the cortical cringe response, whereas the second peak matches the polarized F-actin response (Figure 3D). Under these conditions, after 2 min of stimulation, 67% of cells are polarized, whereas 25% show high cortical F-actin staining (Figure 3D). Remarkably, treatment with either MK886 or LY223982 specifically ablates the second F-actin (Figure 3C). Indeed, after 2 min of stimulation, we found that MK886 and LY223982 treatments decreased the percentage of polarized cells to only 34% and 26%, respectively (Figure 3D). Not surprisingly, we also found that the extent of F-actin accumulation following sub- and saturating IL-8 stimulations, which only lead to low LTB$_4$ secretion (Figure S3A) (Meliton et al., 2010), is not altered in the presence of LTB$_4$ pathway inhibitors (Figures S3B and S3C). These data demonstrate that LTB$_4$ secretion facilitates and stabilizes neutrophil polarization in response to subsaturating stimulations of fMLP. Under these conditions we propose that the limited MyoII phosphorylation measured is a consequence of the absence of cell polarization; we did not observe neutrophil back-retraction defects.

We next wanted to assess if the effects of LTB$_4$ on fMLP-mediated neutrophil polarization were mediated in an autocrine or paracrine fashion. To answer this question, we plated neutrophils at decreasing densities, which gradually reduces the effects of any paracrine signals, and measured the extent of neutrophil polarity 2 min after the addition of 1 nM fMLP. We observed a significant decrease in the percentage of polarized cells as we decreased cell density (Figure 3E), suggesting that

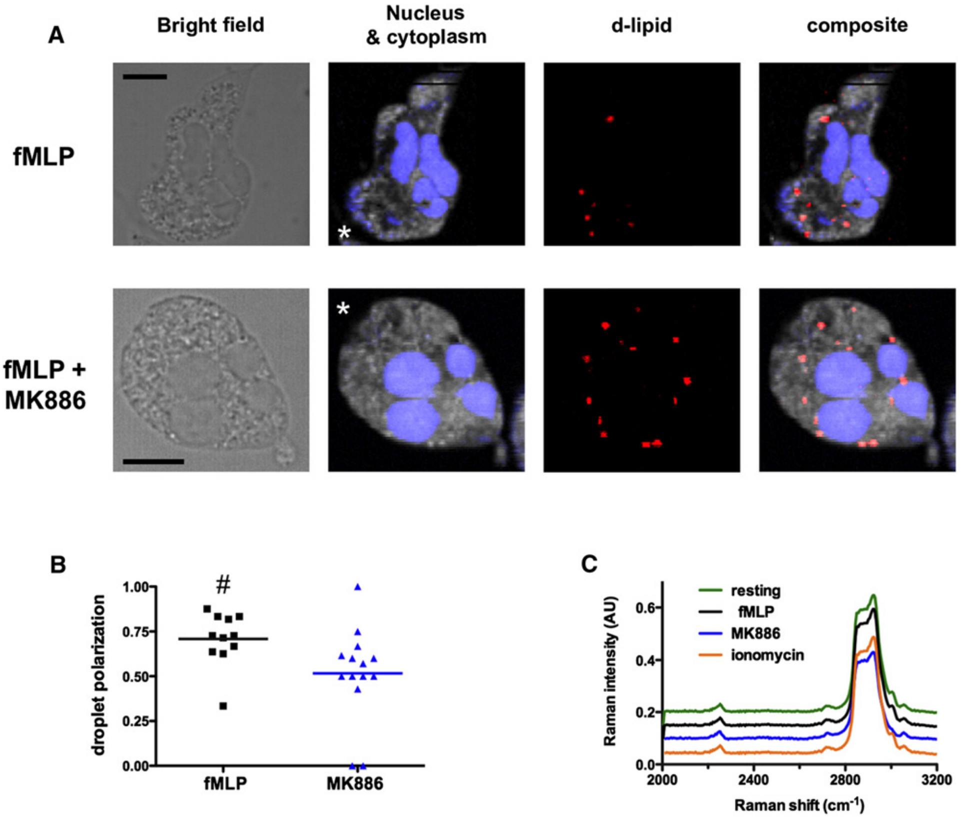

Figure 4. AA Accumulates at the Front of Polarized Neutrophils

(A) Bright-field and CARS images of deuterated punctates localized in polarized primary human neutrophils migrating to 1 μM fMLP in under-agarose assay. Representative images of polarized cells untreated (upper panel) or treated with MK886 (lower panel) are presented. The false-colored chemical images for nucleus (blue), cytoplasm (gray), and deuterated punctates (red) were constructed from Raman intensities at 2,952 cm^{-1} and 2,850 cm^{-1} for nucleus, and intensities at 2,900 cm^{-1} for cytoplasm, and 2,250 cm^{-1} for deuterated punctates, respectively. The asterisks represent the direction of migration. The scale bar represents 5 μm.

(B) Location parameters of deuterated punctates in neutrophils are plotted for two differently treated neutrophils. The location parameter is defined as (number of punctates at the front)/(total number of punctates). Neighboring image pixels (greater than four pixels) are counted as one regardless of the overall size. # $p = 0.009$, Wilcoxon test.

(C) Comparison of the CARS spectra of deuterated punctates found in polarized neutrophils under the indicated conditions.

See also Figure S4.

a paracrine signal regulates neutrophil polarization in response to fMLP. Because this effect is markedly inhibited in the presence of MK886 or LY223982, we propose that LTB$_4$ acts as the main paracrine factor in this response. With 1 nM fMLP stimulations, the paracrine effect is lost when neutrophil density is lower than 10^5 cells/cm^2 because no further decrease in the percentage of polarized cells is observed at 10^5 and 0.5×10^5 cells/cm^2. However, at these cell densities, treatment with either LTB$_4$ pathway inhibitor still significantly reduces the proportion of polarized cells (Figure 3E), suggesting that LTB$_4$ also acts in an autocrine fashion. Taken together, these data demonstrate that, at subsaturating fMLP concentrations, secreted LTB$_4$ functions as a paracrine and autocrine signal to enhance and stabilize neutrophil polarization.

AA Accumulates at the Front of Polarized Neutrophils

We next set out to determine if LTB$_4$ secretion is directionally biased in polarized neutrophils. However, intracellular LTB$_4$ has never been detected in neutrophils stimulated with either fMLP or ionomycin (a major 5-LO activator) (Mita et al., 1988; Williams et al., 1985), suggesting that LTB$_4$ does not accumulate to significant levels in neutrophils. To circumvent this issue, we assessed the subcellular localization of the LTB$_4$ precursor, AA, in polarized neutrophils using coherent anti-Stokes Raman scattering (CARS) microscopy.

Cells pretreated with deuterated AA were allowed to polarize and migrate directionally to fMLP using the under-agarose assay, fixed and analyzed by CARS to determine the subcellular distribution of deuterated species. We detected characteristic spectra for cytoplasm, nucleus, and deuterated punctates (Figure S4A). The peak at ≈2,250 cm^{-1} is characteristic of carbon-deuterium (C-D) bound, whereas the broad peaks at ≈2,900 cm^{-1} are a signature of carbon-hydrogen (C-H) bounds. Remarkably, we found that deuterated punctates accumulate toward the leading edge of neutrophils during chemotaxis (Figures 4A and 4B). In sharp contrast the inhibition of LTB$_4$ synthesis with MK866 rendered the distribution of AA deuterated punctates random (Figures 4A and 4B). Importantly, these findings were not a consequence of the weak cellular polarization measured in the presence of LTB_4 pathway inhibitors because similar findings were obtained when deuterated punctates were monitored following a uniform stimulation with a saturating dose of fMLP (1 μM), which gives rise to normal polarization.

We next compared the averaged spectrum of the deuterated punctates of untreated and MK886-treated cells and found no difference between the two conditions (Figure 4C; see also Figure S4B for a zoomed-in view of the spectra of the C-D bound), even though simulations suggest that the CARS spectra of deuterated AA and deuterated LTB_4 should be different (Figure S4C). Similarly, no deuterated LTB_4 signal could be identified in neutrophils stimulated for longer periods (data not shown) or stimulated with the potent activator of 5-LO, ionomycin (Figures 4C and S4B) (Ford-Hutchinson et al., 1980). It, therefore, appears that, as previously suggested (Mita et al., 1988; Williams et al., 1985), LTB_4 does not accumulate in migrating neutrophils.

We see two possible interpretations of our data: (i) AA is enriched at the front of polarized neutrophils because most of the AA at the back of cells has been converted into LTB_4, which is then secreted at the cell rear; or (ii) AA is relocalized at the front of neutrophils in response to 5-LO activation. Interestingly, we measured the asymmetrical distribution of deuterated punctates in neutrophils as early as 1 min after a uniform stimulation with 1 nM fMLP (data not shown), before the peak of LTB_4 secretion (Figure S4D). This finding suggests that AA is actively redistributed to the front of neutrophils and that LTB_4 is not primarily generated and secreted at the back of cells.

LTB_4 Autocrine/Paracrine Secretion Amplifies Neutrophil Chemotaxis to fMLP

Because cellular polarization is a prerequisite for migration, we studied the role of LTB_4 paracrine/autocrine secretion in neutrophil chemotaxis. We found that treating neutrophils with either MK886 or LY223982 significantly reduces transwell migration to fMLP (Figure 5A). Not surprisingly, neutrophil migration to IL-8 (which induces a very low LTB_4 secretion; Figure S3A) is not altered in the presence of LTB_4 pathway inhibitors (Figure S3D). This finding also confirms that the LTB_4 inhibitors used are specific and do not directly impact neutrophil migration.

This finding was further investigated using the under-agarose assays, where the behavior of populations of cells can be visualized directly (Heit and Kubes, 2003) (Figure 5B). We found that treatment with LTB_4 pathway inhibitors drastically reduces neutrophil chemotaxis to fMLP compared to untreated cells. The inhibition is statistically significant and more dramatic when cells migrated toward lower concentrations of fMLP (Figures 5B and 5C). The reduction in neutrophil chemotaxis in this assay could arise because cells cannot penetrate under the agarose in the absence of LTB_4 signaling or because fMLP-induced LTB_4 secretion amplifies chemotaxis. To get at this, we measured the extent of directed migration as a function of time; we found that in response to either 500 nM or 1 μM fMLP, LTB_4 pathway inhibitors give rise to a gradual inhibition of migration (Figures 5D and 5E), which is indicative of a chemotactic defect. Indeed, if cells were unable to migrate under the agarose, we would expect the migration profiles to show a time delay but otherwise be similar.

It has been shown that fMLP gradients in under-agarose assays are neither linear nor stable over time (Udén et al., 1986). We took advantage of this to study how a neutrophil population migrates into different gradients by assessing the migration speed of cells as a function of the chemoattractant gradient (Figure S5). We found that when neutrophils migrate in either shallow (lower than 25 pM/μm) or steep (greater than 60 pM/μm) gradients, the inhibition of LTB_4 has no significant impact on group migration (Figure 5F). Interestingly, when neutrophils migrate in intermediate gradients (between 25 and 60 pM/μm), the population migrates more efficiently, i.e., the front of migration progresses faster toward the well containing fMLP, in the presence of LTB_4 paracrine/autocrine secretion (Figure 5F). These data are consistent with the fact that fMLP-induced LTB_4 secretion impacts cell polarization at subsaturating (more physiological) concentrations of primary chemoattractants. More importantly, the data highlight the fact that LTB_4 paracrine/autocrine secretion is effective under conditions where LTB_4 is produced in sufficient amounts (i.e., in response to >20 pM/μm) and not overwhelmed by the high concentration of primary chemoattractant (>60 pM/μm).

LTB_4 Paracrine Secretion Acts as a Signal Relay between Neutrophils

We showed that fMLP-induced LTB_4 secretion favors neutrophil polarization and chemotaxis in shallow primary chemoattractant gradients. Several models could explain these observations. First, LTB_4 could increase the capacity of neutrophils to sense fMLP, e.g., by enhancing expression of the fMLP receptor. Second, LTB_4 could act as a chemokinetic agent and simply increase neutrophil migratory capacity. Finally, LTB_4 secretion could form a secondary gradient that facilitates a directional recruitment of neighboring neutrophils. In order to test these possibilities, we took advantage of the availability of mice that lack the formyl receptor 1 (FPR1), which mediates neutrophil chemotaxis to fMLP (Gao et al., 1999), and tested the ability of neutrophils isolated from these mice to migrate to exogenous fMLP when mixed with neutrophils isolated from wild-type (WT) mice.

We first demonstrated that the importance of LTB_4 secretion in neutrophil migration to formyl peptides is not restricted to human primary neutrophils. Using the under-agarose assay, we found that MK886 treatment reduces mouse bone marrow neutrophil migration to the synthetic WKYMVm peptide (a strong agonist for the mouse neutrophil FPR; He et al., 2000) (Figure 6A). Similarly to human neutrophils (Figure 5C), the inhibition is more important for cells migrating to low concentrations of the peptide (Figure 6A). Moreover, we demonstrate that this is not a consequence of drug-induced toxicity in neutrophils: neutrophils isolated from the bone marrow of mice lacking either BLT1 (*blt1*$^{-/-}$) (Tager et al., 2000) or 5-LO (*alox5*$^{-/-}$) (Chen et al., 1994) exhibit impaired migration to 100 nM WKYMVm similarly to what we measure in neutrophils isolated from WT animals treated with MK886 (Figure 6B). Not surprisingly, we also confirmed that neutrophils isolated from *fpr1*$^{-/-}$ mice do not respond to 100 nM MKYMVm (Figure 6B). Importantly, these cells are able to migrate efficiently to LTB_4 (data not shown).

We then mixed cell populations (1:1 ratio) and measured their ability to migrate directionally to MKYMVm using the

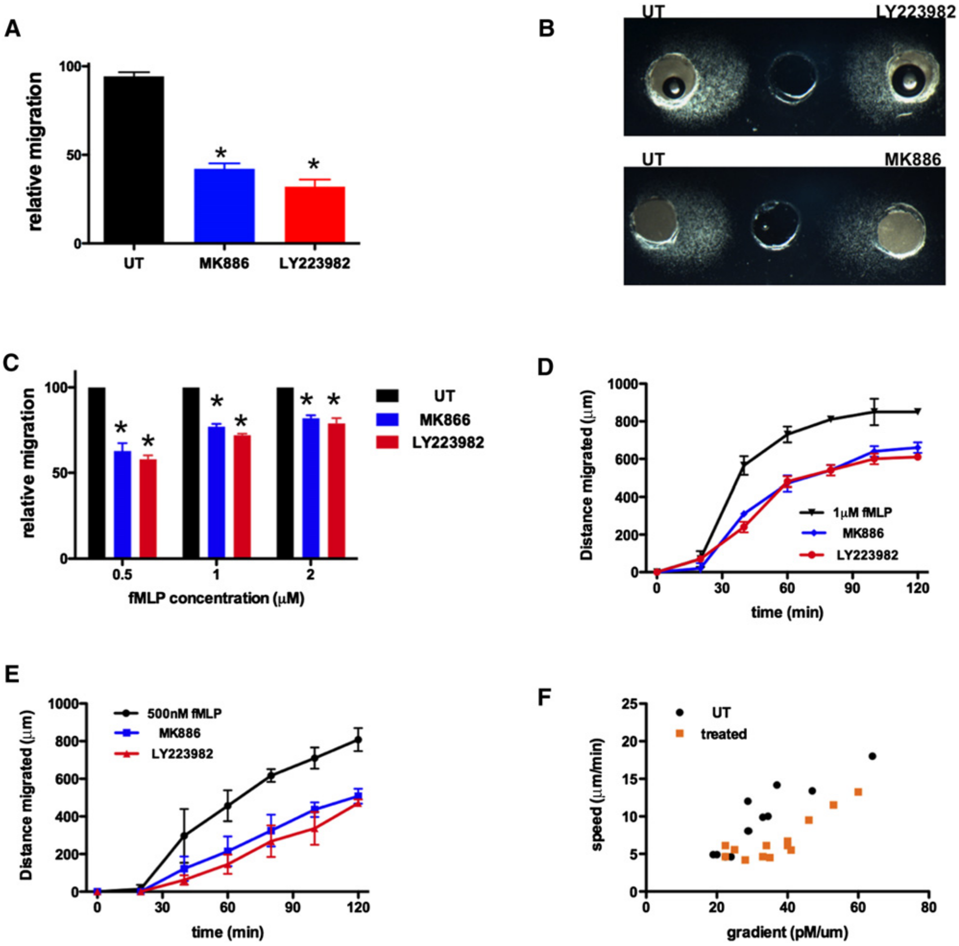

Figure 5. Neutrophil Migration to fMLP Is Amplified by fMLP-Induced LTB$_4$ Paracrine/Autocrine Secretion

(A) LTB$_4$ secretion amplifies neutrophil migration to fMLP. The number of primary human neutrophils migrating to 1 μM fMLP in a 4 μm transwell was determined after 2 hr. Results represent the relative percentage of migrating cells after treatment (average ± SEM) of three independent experiments. $^*p < 0.05$, Friedman test; Dunn's post hoc test.

(B) LTB$_4$ secretion amplifies neutrophil chemotaxis to fMLP. Representative images of primary human neutrophils migrating to 1 μM fMLP in the under-agarose assay are shown.

(C) LTB$_4$ secretion amplifies neutrophil chemotaxis to fMLP. The distance migrated by primary human neutrophils treated with LTB$_4$ pathway inhibitors is compared to the one migrated by untreated cells. Results represent the relative distance migrated (average ± SEM, n = 3) in under-agarose assay in 2 hr. $^*p < 0.05$, Friedman test; Dunn's post hoc test.

(D and E) Kinetics of neutrophil migration in under-agarose assays. The distance migrated by primary human neutrophils to either 1 μM fMLP (D) or 500 nM fMLP (E) was determined at different times points. Results represent the average ± SEM of three independent experiments.

(F) Impact of LTB$_4$ secretion on neutrophil migration to different fMLP gradients. For each segment of 20 min of migration, the average speed was determined, and the local gradient of the front of migration was determined using theoretical charts (see Figure S5). The resulting different data points (speed versus gradient) are plotted.

under-agarose assay. To distinguish between the different populations, mutant neutrophils were fluorescently labeled. We first confirmed that the fluorescent label does not alter neutrophil migration (Figure 6C). Interestingly, we found that, in the presence of neutrophils derived from WT animals, *fpr1*$^{-/-}$ neutrophils gain the capacity to migrate directionally to a well containing MKYMVm. Most importantly, the recruitment of *fpr1*$^{-/-}$ neutrophils is abolished when WT neutrophils are treated with MK886 and do not produce LTB$_4$. Similarly, *fpr1*$^{-/-}$ neutrophils are not recruited when mixed with neutrophils isolated from the bone marrow of *alox5*$^{-/-}$ mice (Figure 6C).

Together, these findings establish that LTB$_4$ acts as a signal-relay molecule for neutrophils where WT neutrophils release LTB$_4$ in an autocrine/paracrine fashion, which provides spatial information to neighboring *fpr1*$^{-/-}$ neutrophils. This LTB$_4$ relay allows the *fpr1*$^{-/-}$ neutrophils to migrate directionally to a chemoattractant they cannot sense.

DISCUSSION

LTB$_4$ is widely recognized as an essential mediator in inflammation. Inhibiting leukotriene production reduces leukocyte recruitment and inflammation in a variety of models, such as arthritis, pancreatitis, or asthma (Peters-Golden and Henderson, 2007). Here, we establish that LTB$_4$ is not only a secondary chemoattractant for neutrophils secreted early in the

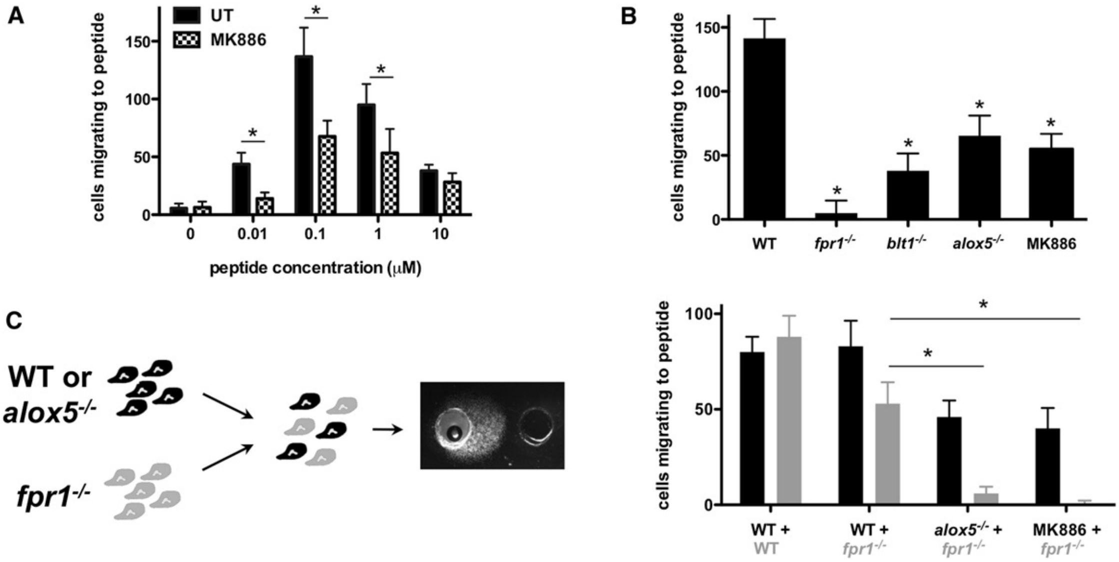

Figure 6. LTB$_4$ Is a Signal-Relay Molecule for Neutrophils

(A) MK886 treatment regulates murine neutrophil migration to MKYMVm. Neutrophils isolated from the bone marrow of WT mouse were allowed to migrate to MKYMVm in the under-agarose assay. The number of neutrophils migrating to fMLP was determined after 5 hr of migration. Results represent the average ± SEM number of migrating mouse neutrophils in three independent experiments. *$p < 0.05$, Mann-Whitney U test.

(B) LTB$_4$ secretion amplifies murine neutrophil migration to MKYMVm. Neutrophils isolated from the bone marrow of mice were allowed to migrate to 100 nM MKYMVm in the under-agarose assay. The number of neutrophils migrating was determined after 5 hr of migration. Results represent the average ± SEM number of migrating mouse neutrophils in three independent experiments. *$p < 0.05$, Friedman test; Dunn's post hoc test.

(C) Neutrophils that do not sense MKYMVm can still migrate to MKYMVm when mixed with WT neutrophils secreting LTB$_4$. Neutrophils isolated from *fpr1*$^{-/-}$ mice were fluorescently labeled and mixed with WT neutrophils (pretreated or not with 100 nM MK886) or neutrophils isolated from *alox5*$^{-/-}$ mice. The number of fluorescent and nonfluorescent cells that migrate to 100 nM MKYMVm was determined after 5 hr migration. Results represent the average ± SEM number of migrating mouse neutrophils in three independent experiments. *$p < 0.05$, Friedman test; Dunn's post hoc test.

inflammation process, but it is also an important signal-relay molecule that increases the recruitment range and promotes the directional migration of neutrophils to formyl peptides, which are released at the core sites of inflammation (McDonald et al., 2010).

We demonstrate that LTB$_4$ relay amplifies cAMP production, MyoII phosphorylation, F-actin polymerization, and cell polarization when cells are stimulated with subsaturating doses of fMLP. This is reminiscent to what has been described in the social amoebae *Dictyostelium discoideum*, where efficient effector activation requires the autocrine/paracrine production of chemoattractants when cells are stimulated with subsaturating concentrations of chemoattractant (Das et al., 2011). By contrast, and similar to the *Dictyostelium* model, LTB$_4$ secretion has no impact on effector activation when neutrophils are stimulated with saturating concentrations of fMLP. These findings also support previous findings (Rochon and Frojmovic, 1993; Tomhave et al., 1994), where at saturating concentrations of fMLP, FPR1 activation induces BLT1 desensitization. Furthermore, we found that LTB$_4$ pathway inhibition specifically impacts migration speed at intermediary fMLP gradients. In this case we envision that under very shallow fMLP gradients, LTB$_4$ production is too low to impact fMLP-induced response, whereas under very steep gradients, LTB$_4$ has no impact on migration because of cross-desensitization. We propose that this intermediary window of fMLP concentrations, where LTB$_4$ relay is a key amplifier, may represent physiologically relevant conditions for in vitro studies.

Both BLT1 and FPR1 are coupled to Gαi-$\beta\gamma$ G proteins, and neutrophil migration toward either LTB$_4$ or formyl peptides is pertussis toxin sensitive (Brito et al., 1997). Therefore, one would expect that LTB$_4$ relay amplifies the same signaling pathways as formyl peptides. However, we show that LTB$_4$ relay specifically amplifies signaling pathways leading to F-actin production and MyoII phosphorylation without affecting Akt and Erk1/2 activation. Differences in signaling pathway activation upon formyl peptides and LTB$_4$ stimulation have been previously reported: fMLP-induced chemotaxis has been shown to require P38-MAPK activation, whereas migration to LTB$_4$ is P38 independent (Heit et al., 2002). Similarly, BLT1 activation does not induce H_2O_2 production, whereas FPR1 activation induces a high pertussis toxin-sensitive H_2O_2 production, and β2-integrin upregulation has been reported to be three times higher upon fMLP stimulation compared to LTB$_4$ stimulation (Berger et al., 2002). Several models can be proposed to explain how the activation of a given Gα subunit can result in different functional responses. First, although the functional relevance of $\beta\gamma$ subtypes has yet to be fully appreciated, the Gαi subunits could associate with different $\beta\gamma$ subunits when coupled to different receptors—this has been demonstrated for the muscarinic M4 and somatostatin receptors binding to Gαo (Kleuss et al., 1992, 1993). Second, FPR1 and BLT1 have been reported to partition in different lipid domains at the plasma membrane (Sitrin et al., 2006). In this context we envision that effector molecules and activated receptors could access different lipid domains resulting in the spatial segregation of signal transduction pathways.

fMLP-induced LTB_4 secretion amplifies neutrophil polarization in an autocrine manner. In fact at low cell density, when LTB_4 cannot act as a paracrine factor, fMLP-induced LTB_4 still enhances neutrophil polarization, albeit to a lesser extent. LTB_4 is not the only autocrine factor associated with effective cell polarization. It has been shown that autocrine ATP secretion enhances lamellipodia formation and stabilization in macrophage and neutrophil chemotaxis to C5a and fMLP, respectively (Chen et al., 2006b; Kronlage et al., 2010). Interestingly, the ATP autocrine activity has been associated with its directed release at the leading edge. We provide evidence that LTB_4 could similarly be secreted at the front of neutrophils. We propose that in both cases the asymmetric secretion enhances lamellipod formation and stabilizes cell polarization by creating a local gradient at the leading edge.

In contrast to ATP, however, we also found that LTB_4 acts in a paracrine fashion to enhance recruitment of neutrophils to primary chemoattractants. Previous studies have also suggested that LTB_4 secretion could act as a paracrine effector for efficient neutrophil activation and degranulation in response to LTB_4 or ATP, respectively (Kannan, 2002; Serio et al., 1997). We predict that both in vivo and in vitro, the secondary gradient generated by the secretion of LTB_4 can efficiently recruit a population of neutrophils that may not normally be recruited to sites of inflammation. This is of consequence because human primary neutrophil populations are heterogeneous. For example three distinct neutrophil subsets, which respond differently to infectious agents, have been identified during *Staphylococcus aureus* infection in mice (Tsuda et al., 2004). In this context, neutrophils that can efficiently migrate to formyl peptides would readily secrete LTB_4, thereby recruiting populations of neutrophils that are low responders for formyl peptides but are good LTB_4 responders. Similarly, in *Dictyostelium*, signal relay has been shown to specifically amplify the range of recruitment of neighboring cells to an external chemoattractant allowing cells to maintain directionality over very long distances (McCann et al., 2010).

It remains unclear how the secondary LTB_4 gradient is formed. Due to its small size (molecular weight = 336 Da), LTB_4 would likely diffuse quickly rendering the gradient short lived. We could first argue that LTB_4 is a lipid-derived hydrophobic molecule, which could significantly reduce its diffusion properties. Second, neutrophils could create a more stable gradient by secreting LTB_4 in exosomes. In *Dictyostelium*, signal relay has been proposed to be mediated by the secretion of chemoattractant-containing exosomes (Kriebel et al., 2008), and FLAP-containing exosomes have been detected in neutrophils (Jethwaney et al., 2007). In addition a recent report has demonstrated that macrophages and dendritic cells are capable of secreting LTB_4-producing exosomes (Esser et al., 2010), which can induce granulocyte migration. Hence, we speculate that neutrophils may secrete such exosomes. In this model, neutrophils that migrate to sites of inflammation would recruit additional neutrophils with LTB_4-releasing vesicles. This model is consistent with our current study and others where intracellular LTB_4 has not been detected (Mita et al., 1988; Williams et al., 1985). This suggests that either LTB_4 is secreted quickly out of the cells or that the cytosolic production of LTB_4 is weak. In this latter scenario, LTB_4 production could be contained within extracellular vesicles.

Based on our findings, we propose the following model for LTB_4-mediated signal relay (see Figure 7). In response to a given external formyl peptide gradient, some neutrophils respond, polarize, and release LTB_4 or LTB_4-producing vesicles at their leading edge. The local LTB_4 gradient strengthens and stabilizes cell polarization of the first responders. Because LTB_4 production is fMLP concentration dependent, neutrophils that are closer to the fMLP source will secrete higher amounts of LTB_4. As a consequence, a secondary LTB_4 gradient is formed parallel to the fMLP gradient. Neutrophils that were not initially responsive to fMLP can now sense the secondary gradient of LTB_4 and migrate up this gradient toward the fMLP source, thus amplifying the inflammatory response.

In summary we provide a mechanism where directional cell-to-cell communication regulates neutrophil migration and recruitment to the core of inflammation sites. We envision this mechanism to be important in vivo where the relay of LTB_4 signals would enhance neutrophil recruitment to the inflammation core at the initiation of the process, when low concentrations of primary chemoattractants are released. In addition we predict that LTB_4 relay is poised to maintain the inflammation. In fact it has been shown that in the absence of LTB_4 signaling, experimentally induced arthritis subsides faster (Chen et al., 2006a; Chou et al., 2010). We propose that in these models, directed neutrophil recruitment to the core of inflammation is enhanced by LTB_4 signal relay.

EXPERIMENTAL PROCEDURES

Additional information is found in the Supplemental Experimental Procedures.

Materials

Percoll, Histopaque 1077, formyl peptides (fMLP for human neutrophils, and the synthetic WKYMVm peptide for mouse neutrophils), IL-8, ionomycin, and LY294002 were obtained from Sigma-Aldrich (St. Louis). LTB_4, deuterated AA, the FLAP inhibitor MK886, and the LTB_4 receptor antagonist LY223982 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-p-Akt (clone C31E5E and D9E for residues T308 and S473, respectively), anti-phosphorylated myosin light chain 2 (Ser19), and anti-p-Erk1/2 (clone D13.14.4E) rabbit antibodies were all from Cell Signaling Technology (Beverly, MA, USA). Transwell chambers were purchased from Corning Life Sciences (Lowell, MA, USA). WT, *alox5*$^{-/-}$, and *blt1*$^{-/-}$ mice were from the Jackson Laboratory (Bar Harbor, ME, USA). *Fpr1*$^{-/-}$ mice were a generous gift from Philip Murphy (National Institute of Allergy and Infectious Diseases, National Institutes of Health [NIH]).

Isolation of Human Peripheral Blood Neutrophils

Heparinized whole blood was obtained by venipuncture from healthy donors. Neutrophils were isolated by dextran sedimentation (3% dextran/0.9% NaCl) coupled to differential centrifugation over Histopaque 1077 (Mahadeo et al., 2007). Residual erythrocytes were removed using hypotonic lysis with 0.2% and 1.6% saline solutions. Blood samples were obtained from anonymous blood donors enrolled in the NIH Blood Bank research program.

Isolation of Mouse Bone Marrow Neutrophils

Mice were sacrificed, and the femurs and tibias were removed from both legs. HBSS (without calcium and magnesium) with 0.1% BSA was forced through the bones, and the solution was filtered trough a cell strainer. Cells were centrifuged at 400 × *g* for 5 min, and neutrophils were isolated using a three-layer Percoll gradient of 78%, 69%, and 52%, as previously described (Boxio

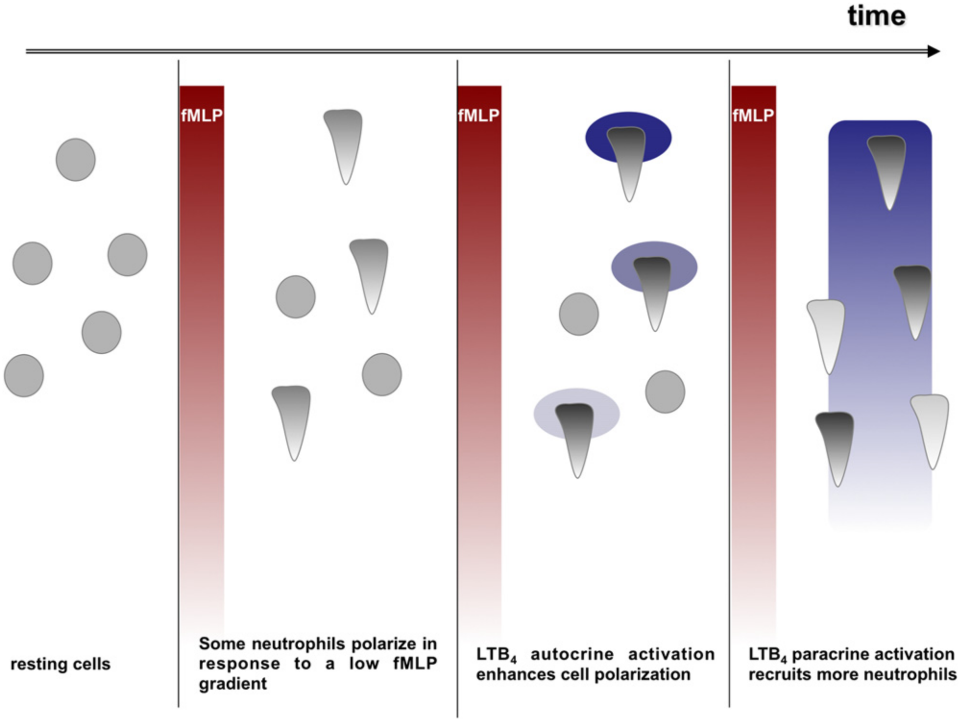

Figure 7. Model for LTB$_4$ as a Signal-Relay Molecule for Neutrophils Migrating to fMLP

In response to an external fMLP gradient, some neutrophils respond, polarize, and release LTB$_4$. The local LTB$_4$ gradient strengthens and stabilizes cell polarization of these first responders. Because LTB$_4$ production is fMLP concentration dependent, a secondary LTB$_4$ gradient is formed parallel to the fMLP gradient. Neutrophils that were not initially responsive to fMLP sense the secondary gradient of LTB$_4$ and migrate up this gradient toward the fMLP source, thus amplifying the inflammatory response.

et al., 2004). After isolation, neutrophils were resuspended in HBSS with or without 1 μM cytotracker green (Molecular Probes; Invitrogen, Eugene, OR, USA), incubated for 1 hr at 37°C, washed, resuspended in RPMI with 10% serum, and incubated for 1 hr at 37°C. Animal procedures were done under protocols approved by the National Cancer Institute, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and policies established by the NIH.

LTB$_4$ Measurement

LTB$_4$ was measured using an ELISA kit (R&D Systems, Minneapolis). Human primary neutrophils were resuspended at 1×10^6 cells/ml in PBS and incubated for 30 min on ice. GM-CSF (10 ng/ml; R&D Systems) was added, and neutrophils were further incubated for 1 hr at 37°C. Cells were spun down at 400 × *g* for 5 min and resuspended (cell density = 15×10^6 cells/ml) with RPMI and incubated at 37°C until stimulated. After the stimulation, cold PBS was quickly added, neutrophils were centrifuged, and supernatants were collected and frozen. Assays were performed according to manufacturer's instructions.

Under-Agarose Assay

Chemotaxis of the neutrophil population was studied using the under-agarose assay as previously described (Comer and Parent, 2006). Cell culture dishes were coated with 1% BSA in PBS for 1 hr at 37°C. For assays with human peripheral blood neutrophils, 0.5% agarose in 50% PBS-50% mHBSS was poured and allowed to solidify for 40 min. For assays with mouse bone marrow neutrophils, 1.2% agarose in 50% PBS-40% RPMI-10% FBS was used. Three 1-mm-diameter wells were carved at 2 mm distance from each other. A chemoattractant was placed in the middle well 15 min before plating neutrophils. A total of 5×10^5 neutrophils in 5 μl mHBSS was plated in the outer wells and incubated at 37°C. Human peripheral blood neutrophils were allowed to chemotax for 2 hr unless otherwise mentioned, and mouse bone marrow neutrophils were allowed to chemotax for 5 hr. The wells with human peripheral blood neutrophils were visualized using a Leica DM IL stereoscope. Assays using human peripheral blood neutrophils were quantified using ImageJ by measuring the distance the cells had migrated directionally toward the chemoattractant. For the mouse bone marrow neutrophils, fluorescent cells were counted using a Zeiss Axiovert S100 epifluorescent microscope.

CARS Microscopy

Neutrophils were incubated with deuterated AA as reported previously (van Manen et al., 2005). Labeled cells were allowed to migrate in under-agarose assay for 2 hr, or stimulated uniformly for 1 or 2 min with 10 nM fMLP. The experimental setup of the broadband CARS microscopy has been described previously (Lee et al., 2011; Parekh et al., 2010). Briefly, the output (70 fs, centered at 830 nm, 80 MHz) of a Ti:S laser oscillator (MaiTai-DeepSee, Spectra-Physics) was split into two parts. One part was introduced into a photonic crystal fiber (Crystal Fibre; FemtoWHITE) to generate a continuum pulse. The other part was spectrally narrowed by a 4-*f* dispersion-less filter to 10 cm^{-1} full-width half-maximum (FWHM) with the center wavelength at 830 nm. The two beams were introduced collinearly and with parallel polarization into a 60× 1.35 NA oil-immersion objective lens (Olympus) and focused on the sample. The CARS signal generated from the sample was collected in the forward direction and passed through a set of an 830 nm notch filter and an 810 nm short-pass filter and was analyzed using a charge-coupled device (CCD; DU920-BR-DD; Andor) attached to a monochromator (SP-2300; Acton). The spatial resolution was laterally 500 nm, and the sample was scanned either by 120 or 250 nm pixel spacing. The average laser power at the sample was kept below 15 mW for each pulse to avoid photodamage. The CCD exposure time is typically 30 ms per pixel. The acquired CARS spectrum was processed by modified Kramers-Kronig phase retrieval and followed by baseline detrending (Liu et al., 2009).

Statistical Analysis

Analyses were performed with GraphPad Prism software Version 5.0b. One-way ANOVA and Dunnet post hoc test (with untreated cells as the control group) were performed on normalized data with “treatment” as the independent variable and “cAMP level,” “F-actin accumulation,” or “number of cells migrating in a transwell assay” as the dependent variable ($p < 0.05$ was considered statistically significant). Friedman and Dunn’s post hoc test (with untreated cells as the control group) was performed with “treatment” as the independent variable and “normalized MyoII phosphorylation levels” or “distance migrated in under-agarose assay” as the dependent variable ($p < 0.05$ was considered statistically significant). For the asymmetrical distribution we compared the distribution to a theoretical 0.5 mean value in a Wilcoxon test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2012.02.003.

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