The nuclear membrane leukotriene synthetic complex is a signal integrator and transducer

Angela M. Bair*, Melissa V. Turman*, Christine A. Vaine*, Reynold A. Panettieri, Jr.#, and Roy J. Soberman*
*Renal Unit, Department of Medicine, Massachusetts General Hospital, Charlestown, MA 02129; #Airways Biology Initiative, Pulmonary, Allergy, and Critical Care Division, University of Pennsylvania, Philadelphia, PA 19104

ABSTRACT Leukotrienes (LTs) are lipid-signaling molecules derived from arachidonic acid (AA) that initiate and amplify inflammation. To initiate LT formation, the 5-lipoxygenase (5-LO) enzyme translocates to nuclear membranes, where it associates with its scaffold protein, 5-lipoxygenase–activating protein (FLAP), to form the core of the multiprotein LT synthetic complex. FLAP is considered to function by binding free AA and facilitating its use as a substrate by 5-LO to form the initial LT, LTA4. We used a combination of fluorescence lifetime imaging microscopy, cell biology, and biochemistry to identify discrete AA-dependent and AA-independent steps that occur on nuclear membranes to control the assembly of the LT synthetic complex in polymorphonuclear leukocytes. The association of AA with FLAP changes the configuration of the scaffold protein, enhances recruitment of membrane-associated 5-LO to form complexes with FLAP, and controls the closeness of this association. Granulocyte monocyte colony–stimulating factor provides a second AA-independent signal that controls the closeness of 5-LO and FLAP within complexes but not the number of complexes that are assembled. Our results demonstrate that the LT synthetic complex is a signal integrator that transduces extracellular signals to modulate the interaction of 5-LO and FLAP.

INTRODUCTION

The mammalian innate immune system is poised to respond quickly to bacterial infection and tissue injury, with polymorphonuclear leukocytes (PMNs) being the initial responders. Not only do PMNs provide the first line of host defense to bacteria, but they also amplify the inflammatory response by producing cytokines, chemokines, and the lipid-signaling molecule leukotriene (LT) B4 (Borgeat and Samuelsson, 1979; Scapini et al., 2001; Cloutier et al., 2009). LTB4 is a product of the 5-lipoxygenase (5-LO) pathway of arachidonic acid (AA) metabolism and initiates and amplifies the innate immune response by functioning as a highly potent chemoattractant for PMNs, monocytes, mast cells, and certain T-cell subsets (Ford-Hutchinson et al., 1989; Showell et al., 1995). Recently Afonso et al. (2012) showed that LTB4 plays a key role in controlling cellular responses to formyl peptide–mediated neutrophil polarization, resulting in augmented chemotaxis and an appropriate response to infectious and inflammatory challenges. Therefore cells must be able to both integrate extracellular signals to generate LTB4 and transduce these signals to yield a flexible and appropriate LT response. How they achieve this is not known.

To prevent the unwanted initiation of inflammation and ensure that an inflammatory response is appropriately matched to a given challenge, cells have evolved a complex series of cellular and molecular controls to regulate LT synthesis (Figure 1). In resting cells, 5-LO, the initial enzyme of the LT pathway, resides in the cytosol and transduces extracellular signals to generate AA-dependent and AA-independent signals that control the synthesis of AA-dependent and AA-independent LTs. The nuclear membrane leukotriene (NML) complex is a signal integrator that transduces extracellular signals to modulate the interaction of 5-LO and FLAP.
activation, increased intracellular calcium triggers the translocation of cPLA2 to the endoplasmic reticulum (ER), outer nuclear membrane, and the Golgi (Glover et al., 1995; Evans et al., 2001; Leslie, 2004). In parallel, 5-LO translocates to the inner and outer nuclear membranes in a calcium-dependent process (Rouzer and Kargman, 1988; Rouzer et al., 1989; Woods et al., 1993; Kulkarni et al., 2002). The N-terminal C2 domain of cPLA2 (Nalefski et al., 1994; Evans et al., 2004) and C2-like domain of 5-LO (Kulkarni et al., 2002) mediate their association with specific membrane phospholipids. Although cPLA2 efficiently releases AA, the integral membrane protein 5-lipoxygenase–activating protein (FLAP) is required for the efficient conversion of endogenous AA to LTA4 (Miller et al., 1990; Reid et al., 1990). X-ray crystallography identified FLAP as a trimer (Ferguson et al., 2007). FLAP contains four transmembrane helices that are connected by two cytosolic loops and one loop facing the lumen. FLAP is localized to the inner and outer nuclear membranes and also ER (Dixon et al., 1990; Miller et al., 1990). FLAP is considered to function as a scaffold protein that brings AA in sufficient proximity with 5-LO to allow efficient metabolism of AA.

Prior findings from our laboratory indicate that 5-LO directly interacts with FLAP in activated cells to form the core of a multiprotein LT synthetic complex (Mandal et al., 2008). To initiate LT synthesis, 5-LO converts AA to 5-hydroperoxycisotetraenoic acid, which is then converted to LTA4 (Rouzer et al., 1986). In some myeloid cell types such as mast cells, LTA4 is converted to LTC4 by the enzyme LTC4 synthase (Penrose et al., 1992; Lam et al., 1994). LTC4 synthase is an integral membrane protein that is tightly associated with FLAP in the outer nuclear membrane (Mandal et al., 2004). Alternatively LTA4 is subsequently converted to LTB4 by LTA4 hydrolase, which is present in the cytosol of PMNs (Radmark et al., 1984).

It is not known how multiple extracellular signals from cytokines, chemokines, lipid mediators, and C5a are integrated in the correct temporal and spatial order to assemble LT synthetic complexes and generate an appropriately graded LTB4 output. PMNs are a useful cell to address this question because they require two distinct signals for optimal LTB4 synthesis (Dahinden et al., 1988; Krump and Borgeat, 1994). The complement component C5a signals through its cognate G protein–coupled receptor (GPCR) to give an increase in intracellular calcium, leading to the translocation of 5-LO and cPLA2 to the nuclear envelope and the subsequent conversion of AA to a small but significant amount of LTB4 (Dahinden et al., 1988; Krump and Borgeat, 1994). Pretreatment, or “priming,” of PMNs with a cytokine such as granulocyte macrophage-colony-stimulating factor (GM-CSF) augments C5a-dependent LTB4 synthesis by threefold to fourfold, even though no LTB4 is produced in response to GM-CSF treatment alone (DiPersio et al., 1988; Krump and Borgeat, 1994; Brock et al., 1996). Because priming is not simply an additive effect, various mechanisms have been proposed to explain the process. Phosphorylation of cPLA2 at Ser-505 enhances its activity, leading to increased AA availability (McColl et al., 1991; Lin et al., 1993). Phosphorylation of 5-LO has been suggested to play a role in regulating activity (Wertz et al., 2002), but these observations were made using nonphysiologic conditions. No previous studies have addressed the concept that discrete extracellular signals might alter macromolecular interactions in the LT synthetic complex.

Although the steps leading to the assembly of the LT synthetic complex and LT synthesis are well established (Figure 1), two key questions remain regarding the assembly of 5-LO and FLAP on the nuclear envelope: 1) Is the association of 5-LO with the nuclear membrane sufficient to promote binding to FLAP, or are additional signals required to initiate this protein–protein interaction? 2) Does free AA released from phospholipids modulate LT synthetic complex formation? We used fluorescence lifetime imaging microscopy (FLIM) to define a multistep assembly process for formation of LT synthetic complexes on the nuclear envelope. We identified distinct AA-dependent and AA-independent steps that control the interaction of 5-LO and FLAP. Overall, we identify the LT synthetic complex as a regulatory structure that functions by integrating and transducing diverse extracellular signals to regulate the interaction of 5-LO with FLAP. These qualitative changes are directly associated with levels of the cellular LTB4 response to cell activation by combinations of C5a and GM-CSF.

**RESULTS**

Multiple signals regulate the assembly of the LT synthetic complex in PMNs

We sought to define the multiple potential steps in assembling the LT synthetic complex. We confirmed the priming effect of GM-CSF in mature PMNs isolated from mouse bone marrow. Stimulation of PMNs with 100 nM C5a for 5 min induced a small but significant amount of LTB4 generation (Figure 2). Preincubation with 1 ng/ml GM-CSF (~70 pM) for 60 min augmented the generation of LTB4 by PMNs in response to stimulation with C5a by threefold to fourfold. Neither unstimulated cells nor cells only primed with GM-CSF generated detectable amounts of LTB4.

Because cPLA2 requires Ca2+ for its association with phospholipids in nuclear membranes (Glover et al., 1995; Evans et al., 2001, 2004), we tested whether the priming effect of GM-CSF could be explained by an increase in Ca2+ flux, potentially leading to an increase in membrane-associated cPLA2 molecules. Cells were primed with GM-CSF, followed by stimulation with C5a. Calcium flux was compared with that seen in cells stimulated with C5a alone. As shown in Table 1, both primed and unprimed PMNs showed a comparable increase in Ca2+ in response to C5a, indicating that differences in Ca2+ levels did not account for the increased LTB4 production with GM-CSF priming. Cells treated with ionomycin (positive
control) showed a marked increase in Ca\(^{2+}\), whereas no change in Ca\(^{2+}\) flux was seen in unstimulated cells.

We hypothesized that differences in LTB\(_4\) synthesis could ultimately be translated into discrete events at the level of membrane organization of the LT synthetic complex. To test this, we evaluated the relationship between 5-LO and FLAP on the nuclear membrane in response to cell stimulation by a combination of epifluorescence and FLIM. Aliquots of cells from each experimental condition were collected in parallel, fixed, permeabilized, and probed with antibodies (Abs) to the C-terminus of 5-LO and to the C-terminus of FLAP, which were detected with Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary Abs, respectively. Because FLAP is an integral membrane protein that is present predominantly in the nuclear membrane of PMNs (Woods et al., 1993), we were able to distinguish nuclear membranes from the nucleoplasm by comparing 4′,6-diamidino-2-phenylindole (DAPI) staining to the localization of FLAP staining. The results are supported by confocal microscopy (Supplemental Figure S1). After cellular activation by C5a alone or by C5a after priming with GM-CSF, 5-LO colocalized with FLAP on the nuclear envelope (Figure 3B).

Cells were also analyzed by FLIM, a quantitative method for measuring Förster resonance energy transfer (FRET, Table 2 and Figure 3C). FLIM allows evaluation of the fluorescence lifetime of a donor molecule, in this case 5-LO, labeled with Alexa Fluor 488–conjugated secondary Abs. After determining the donor fluorescence lifetime in the absence of the acceptor fluorophore (Figure 3C, Donor only), FRET between donor and acceptor was defined by the lifetime of interacting molecules (\(\tau_1\)), with \(a_1(\%\) representing the fraction of interacting molecules. We found that a small percentage of 5-LO molecules interacted with FLAP on the nuclear membrane in unstimulated cells (Table 2), consistent with previously reported findings in unprimed RBL-2H3 cells (Mandal et al., 2008). After stimulation with C5a, \(a_1(\%\) was not significantly different from that in unstimulated cells, but \(\tau_1\) significantly decreased (Table 2 and Figure 3C), indicating increased proximity between 5-LO and FLAP. When cells were primed with GM-CSF before C5a stimulation, a further decrease in \(\tau_1\) and a significant increase in \(a_1(\%\) were observed, indicating a closer association and an increase in the number of interactions between 5-LO and FLAP. GM-CSF itself had no effect on \(\tau_1\) (Supplemental Table S1).

**AA-dependent and AA-independent signals control the assembly of 5-LO and FLAP**

Several studies equated the effect of GM-CSF or other cytokines to an increased availability of AA secondary to phosphorylation of cPLA\(_2\) (DiPersio et al., 1988), resulting in increased catalytic activity (McColl et al., 1991; Lin et al., 1993; Krump and Borgeat, 1994). We therefore hypothesized that a subtle rearrangement of the structure of the FLAP trimer occurs upon binding of AA and that this could bring a FLAP domain in closer apposition to the C-terminal catalytic domain of 5-LO; this would manifest as an AA-dependent decrease in \(\tau_1\). To test this possibility, we first determined the effect of cPLA\(_2\) inhibitor (cPLA\(_2\) Inh) N-(25,4R)-(4-biphenyl-2-ylmethyl-isobutyramino)-1-2-[2,4-difluorobenzoyl]-benzoyl]-pyrrolidin-2-ylmeth]-8-3-[4-2,4-dioxaethoxidin-5-yldenemethyl]-phenyl]acrylamide) on both LTB\(_4\) synthesis and the interaction between 5-LO and FLAP. Treatment of PMNs with cPLA\(_2\) Inh at concentrations between 100 nM and 10 μM before stimulation significantly inhibited LTB\(_4\) synthesis (Figure 4 and Supplemental Figure S2). Of note, the pyrrilidone cPLA\(_2\) inhibitor used in this study has been shown to have no detectable inhibition of purified, recombinant human sPLA\(_2\)-IIA, group V, or group X sPLA\(_2\) at a concentration of 10 mM and no significant inhibition of either recombinant cytosolic PLAD or the calcium-independent PLAD, iPLA\(_2\) (Ghomashchi et al., 2001; Bryant et al., 2011).

In parallel, we analyzed cells from each of these populations by both epifluorescence and FLIM. Inhibition of AA release by cPLA\(_2\) Inh (5 μM) had no obvious effect on the movement of 5-LO to the nuclear membrane in cells either stimulated directly with C5a or primed with GM-CSF before stimulation with C5a (Figure 5A), suggesting that 5-LO movement to the nuclear membrane is independent of AA release. We next analyzed the interaction between 5-LO and FLAP in the presence and absence of cPLA\(_2\) inhibition. There was a small but quantifiable percentage of 5-LO that interacts with FLAP on the nuclear membrane of unstimulated cells (Table 2). In addition, \(\tau_1\) values remained unchanged upon exposure to GM-CSF alone and cPLA\(_2\) Inh alone (Supplemental Table S1). Also, as noted, cells stimulated with C5a exhibited a decreased \(\tau_1\) compared with unstimulated controls (Table 2). However, when C5a-stimulated cells

| Condition                  | \(\Delta[Ca^{2+}]_\text{p}, \text{ AU}\) | \(\Delta[Ca^{2+}]_\text{p}, \text{ AU}\) |
|----------------------------|----------------------------------------|----------------------------------------|
| Unstimulated               | 0.1 ± 0.2                              |                                          |
| C5a                        | 0.5 ± 0.1                              |                                          |
| GM-CSF + C5a               | 0.7 ± 0.2                              |                                          |
| Ionomycin                  | 3.0 ± 0.4                              |                                          |

Values represent mean ± SEM for three independent experiments. AU, arbitrary units.

**TABLE 1:** Calcium flux in stimulated PMNs.
were pretreated with cPLA₂ Inh, the $\tau_1$ values were similar to those of unstimulated cells. These results strongly support a role for AA in regulating the closeness between 5-LO and FLAP.

A distinct response was observed in cells primed with GM-CSF before C5a stimulation (Table 2 and Figure 5B). In the presence or absence of cPLA₂ Inh, $\tau_1$ decreased relative to values in unstimulated cells. However, cPLA₂ inhibition altered the interaction between FLAP and 5-LO, with $\tau_1$ approaching those of cells treated with C5a alone. This suggests that in addition to the AA-dependent effect observed with C5a, an AA-independent, GM-CSF–dependent process regulates the relationship of 5-LO to FLAP. When cPLA₂ was inhibited, $a_1$(%) values returned to baseline, indicating a role for AA in controlling the number of LT synthetic complexes formed on nuclear membranes.

Two of the major intracellular signaling pathways that are engaged by the activation of the GM-CSF receptor are p38 mitogen-activated protein kinase (MAPK) and extracellular-regulated kinase (ERK; Hercus et al., 2009), each of which has been linked to post-translational modification of enzymes regulating LT synthesis. Both p38 MAPK and ERK have been shown to play redundant roles in the phosphorylation and regulation of cPLA₂ (Geijsen et al., 2000). Furthermore, pharmacological inhibition of ERK has been shown to inhibit LT formation in response to f-Met-Leu-Phe (fMLP; Werz et al., 2002), potentially, in part, by blocking phosphorylation of 5-LO. Inhibition of ERK with 10 μM U0126 completely eliminated LTB₄ production in response to combinations of GM-CSF and C5a (Supplemental Figure S3). When aliquots of the same cells were analyzed for FLIM, the low $\tau_1$ values seen in stimulated cells returned to those seen in unstimulated control cells, whereas $a_1$(%) levels remained elevated when compared with unstimulated controls (Table 3). When the same experiment was performed in the presence of the cPLA₂ inhibitor, in addition to $\tau_1$, the $a_1$(%) values returned those seen in unstimulated cells. These data indicate a role for ERK in controlling the qualitative association of 5-LO and FLAP that is independent of AA.

When p38 MAPK was inhibited in cells by 10 μM SB203580, LTB₄ production was inhibited by ~80% in response to the combination

| Condition | $\tau_1$ (ps) | $a_1$(%) |
|-----------|---------------|-----------|
| Donor only | 3000 ± 1      | 100 ± 0   |
| Unstimulated | 1920 ± 1     | 9.5 ± 1a  |
| C5a       | 1356 ± 74b   | 12 ± 2a   |
| GM-CSF + C5a | 826 ± 89b,c | 29 ± 5b,c |
| cPLA₂ Inh + C5a | 1828 ± 89a | 12 ± 1a  |
| cPLA₂ Inh + GM-CSF + C5a | 1256 ± 187a,b | 9 ± 2a |

Values represent means ± SEM for $n = 20$ for each condition.

$a_p < 0.01$ vs. GM-CSF + C5a.

$b_p < 0.001$ vs. unstimulated cells.

$c_p < 0.0001$ vs. C5a.

**TABLE 2:** FLIM analysis of 5-LO/FLAP interactions.
of GM-CSF and C5a (Supplemental Figure S3). When compared with stimulated cells, an increase in $\tau_1$ was observed; however, there was no change in $a_1(\%)$ (Table 3). With the inclusion of the cPLA$_2$ inhibitor, a further increase in $\tau_1$ was observed, matching what was seen in unstimulated controls. This indicates a role for p38 MAPK independent of AA in regulating the interaction of 5-LO and FLAP. The mechanism by which ERK and p38 function in this process remains to be determined.

**AA controls the relationship between the N- and C-terminal domains of FLAP**

We postulated that AA could function to promote the interaction of 5-LO with FLAP by binding to the scaffold protein and causing a change in the molecular arrangement of FLAP. To monitor this effect, we used FLIM to monitor interactions between the N- and C-termini of FLAP. The N-terminus of FLAP (amino acids 1–19) was identified with Alexa Fluor 488–conjugated secondary antibody (donor fluorophore) and FLAP with Alexa Fluor 594–conjugated secondary antibody (acceptor fluorophore). This approach allowed the detection of potential changes in the relationship between the domains of individual monomers, between monomers of a given trimeric FLAP molecule, or between monomers of adjacent trimers. The relatively low values of $a_1(\%)$ in all samples (Table 4) suggest that the majority of interactions detected between N- and C-termini are not within individual monomers but between either adjacent trimeric FLAP molecules or, possibly, between different FLAP trimers. The most likely reason for this is steric considerations of the relationships between Abs and that the size of FLAP affected analysis of the domains of individual monomers.

In unstimulated cells, the $\tau_1$ value was 543 ± 27 ps (Table 4). When PMNs were stimulated with C5a, $\tau_1$ increased, indicating a significant change in the relationship of the N- and C-termini; this change was not observed when cells were primed with GM-CSF before C5a stimulation. However, the percentage of interacting N- and C-termini decreased after C5a stimulation in both unprimed and primed cells. When cPLA$_2$ was inhibited, $\tau_1$ values were not statistically different from those in unstimulated cells; furthermore, $a_1(\%)$ increased to levels seen in unstimulated cells. Although the mechanism is unclear, these results indicate that AA affects the
DISCUSSION

LT biosynthesis in PMNs requires the dynamic construction of macromolecular complexes on the nuclear membrane. Although prior studies focused on the discrete step of 5-LO translocation to the nuclear envelope and the use of AA as a substrate for generating leukotrienes through the activation of cPLA2 (Nahas et al., 1996), these studies did not address how LT synthesis is organized on the nuclear envelope. We report here that multistep assembly and organization of the LT synthetic complex in isolated murine PMNs involves processes that are both dependent on and independent of AA release. The flexibility of this process and response to different signals supports a role for the LT synthetic complex as the regulator of the qualitative interaction between 5-LO and FLAP, a relationship that is likely to be reflected in the output of LTA4.

To elucidate whether assembly of the LT synthetic complex on the nuclear envelope of PMNs was altered by different signals, we exploited the concept of “priming,” in which a signal by a transmembrane cytokine receptor, such as the GM-CSF receptor, synergistically augments LT synthesis in concert with a GPCR agonist such as C5a (DiPersio et al., 1988). As expected, priming by GM-CSF increased LT synthesis threefold to fourfold even though there was no difference in calcium flux compared with unprimed, stimulated cells.

The additional amplification of LT synthesis imparted by GM-CSF priming has two effects. First, it amplifies the AA-driven assembly of the LT synthetic complex, regulating an increase in the percentage of nuclear membrane–associated 5-LO molecules interacting with FLAP. This is distinct from the effect seen by stimulation with C5a alone. GM-CSF also provides a signal that drives the closeness of the association of 5-LO and FLAP (Figures 3 and 6 and Table 2). The mechanism by which GM-CSF exerts these two distinct activities may be related to the phosphorylation of cPLA2 and 5-LO. Priming by GM-CSF stimulates the MAPK pathway, which can phosphorylate cPLA2 (Geijsen et al., 2000). This would augment the release of AA from membrane phospholipids. Phosphorylation of 5-LO can affect its intracellular trafficking; whether it directly affects the association of 5-LO and FLAP on the nuclear envelope, however, is not known. Both ERK and p38 MAPK inhibition alter the interaction of 5-LO and FLAP independent of AA (Table 3). However, whether this is secondary to phosphorylation of 5-LO or the nuclear membrane–associated molecules interacting with FLAP (Supplemental Table S2).

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**TABLE 3:** FLIM analysis of 5-LO/FLAP interactions.

| Condition | $t_1$ (ps) | $a_1$ (%) |
|-----------|-----------|-----------|
| Donor only | 2900 ± 4 | 100 ± 0  |
| Unstimulated | 1899 ± 30 | 11 ± 9   |
| GM-CSF + C5a | 562 ± 32 | 49 ± 11  |
| 10 μM SB203580 + GM-CSF + C5a | 1635 ± 81 | 32 ± 27  |
| 10 μM U0126 + GM-CSF + C5a | 2003 ± 29 | 28 ± 19  |
| 5 μM cPLA2 Inh + GM-CSF + C5a | 1492 ± 33 | 11 ± 9   |
| 10 μM SB203580 + 5 μM cPLA2 Inh + GM-CSF + C5a | 2001 ± 80 | 6 ± 3    |
| 10 μM U0126 + 5 μM cPLA2 Inh + GM-CSF + C5a | 1960 ± 91 | 9 ± 7    |

Values represent means ± SEM from two separate experiments, where $n=15$ for each condition.

**TABLE 4:** FLIM between the N- and C-termini of FLAP.

| Condition | $t_1$ (ps) | $a_1$ (%) |
|-----------|-----------|-----------|
| Unstimulated | 543 ± 27 | 32 ± 2   |
| C5a | 865 ± 49$^a$ | 16 ± 4$^b$ |
| GM-CSF + C5a | 522 ± 34 | 18 ± 2$^b$ |
| cPLA2 Inh + C5a | 622 ± 30 | 41 ± 2   |
| cPLA2 Inh + GM-CSF + C5a | 568 ± 27 | 41 ± 2   |

FLIM between N-terminus (amino acids 1–19) and C-terminus (amino acids 148–161) is represented by values ± SEM of $n=20$ for each experimental condition. $^a p<0.001$ vs. all other $t_1$, conditions. $^b p<0.001$ vs. unstimulated $t_1$ condition.

**FIGURE 6:** The multistep assembly of the LT synthetic complex.

1) Under resting conditions 5-LO resides in the cytoplasm and FLAP within the nuclear membrane. No LTs are made under this condition.
2) C5a-induced cell activation, followed by a rise in intracellular calcium, redistributes 5-LO to the nuclear membrane in close proximity with FLAP. AA is cleaved from phospholipids and interacts with FLAP; modest amounts of LTA4 are generated. Change in FLAP domains by FLIM are illustrated by the trapezoidal FLAP. 3) Priming cells with GM-CSF followed by C5a activation results in increased 5-LO translocation to the nuclear membrane, tighter interactions with FLAP, and robust LTA4 production. 4) AA controls the assembly of the LT synthetic complex. In the presence of cPLA2 Inh, 5-LO translocates to the nuclear membrane after C5a stimulation but does not show an increase in either the percentage of molecules interacting with FLAP or the “closeness” of its interaction. No LTs are made. 5) Priming cells with GM-CSF controls the interaction of 5-LO and FLAP by AA-dependent and AA-independent mechanisms. Cells treated with cPLA2 Inh and primed with GM-CSF followed by C5a activation show an increase in the closeness of 5-LO and FLAP but not in the percentage of membrane-associated molecules interacting with FLAP. No LTs are produced.
posttranslational modification of a yet-to-be-determined complex member is unknown.

The ability of cells to qualitatively modify the association of 5-LO with FLAP in response to discrete signals has the potential to provide the flexibility to generate the appropriate amount of LTβ4 in different biological settings. This can be illustrated by considering the diverse signaling responses of the GM-CSF receptor (Hercus et al., 2009). At low (femtomole) levels of GM-CSF, the receptor is exclusively phosphorylated at βc Ser-585, engaging PI3 kinase and Akt pathways that do not lead to posttranslational modification of either cPLA2 or 5-LO, and are not known to prime PMNs for augmented LTβ4 synthesis. This would potentially correspond to a setting of early or low-level tissue inflammation or injury, and the generation of LTβ4 would be determined solely by the AA levels induced by C5a, fMLP, or chemokines. In settings of more severe inflammation or infection, higher levels of GM-CSF would be generated. This would lead to exclusive phosphorylation of βc Tyr-577 and extinction of Ser-585 phosphorylation. This engages the MAP kinase pathway, allowing for the phosphorylation of cPLA2 and increased release of free AA. In turn, this would lead to the incorporation of a higher percentage of nuclear membrane–associated 5-LO into LT synthetic complexes with increased closeness between 5-LO and FLAP. Phosphorylation of 5-LO would have an additional effect by increasing the closeness of its association with FLAP. The net effect of these changes would be the generation of more LTβ4 and the recruitment of more PMNs and other LTβ4-responsive cells to the inflamed tissue. The levels of GM-CSF used in this study to prime PMNs are within this higher range.

It has been shown that ligands of scaffold proteins impart an allosteric effect on the scaffold protein's ability to recruit a second ligand (Good et al., 2011). We found that AA has a direct effect on the interaction of the N- and C- termini of FLAP. Furthermore, in the presence of the cPLA2 Inh, primed and unprimed cells treated with C5a exhibited statistically decreased LTβ4 production. We also found that in cells exposed to the cPLA2 Inh and C5a, τ1 values significantly decreased close to those observed in unstimulated cells. Similarly, cells that were incubated with the cPLA2 Inh, primed, and treated with C5a have significantly reduced τ1 values compared with cells primed and treated without the inhibitor. Taken together, these results imply that AA governs the closeness of the association of 5-LO and FLAP.

The use of AA as a means of modulating the LT synthetic complex yields increased flexibility in terms of regulating LT synthesis. Studies have shown that the regulation of the reactivation of free AA is a key step in LT synthesis in PMNs, especially under the conditions of priming (Zarin et al., 2006). Therefore it will be important to understand the regulation of the transacylase enzymes.

In addition to the studies indicating that LTβ4 dramatically amplified formyl peptide–mediated neutrophil chemotaxis and cytoskeletal rearrangement by acting on signaling pathways upstream of actin polymerization (Afonso et al., 2012), C5a, fMLP, and IL-8 all stimulate PMNs to generate LTβ4, and all have LTβ4 synthesis amplified by priming (Dahinden et al., 1988; DiPersio et al., 1988; Krump and Borgeat, 1994; Afonso et al., 2012). Therefore the observations that AA stimulates the assembly of the LT synthetic complex in response to C5a likely represent a general mechanism for transducing and amplifying signaling by chemoattractants.

**MATERIALS AND METHODS**

**Mice**

C57BL/6 mice 8–12 wk old were obtained from Jackson Labs (Bar Harbor, ME) and maintained under specific-pathogen–free conditions at the animal facilities at the Massachusetts General Hospital Charlestown Facility. Experimental manipulations were performed in accordance with Institutional Animal Care and Use Committee recommendations at Massachusetts General Hospital.

**Reagents**

Poly-I-lysine solution was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse GM-CSF was obtained from Millipore (Billerica, MA), and C5a was from R&D Systems (Minneapolis, MN). Vectashield with DAPI was purchased from Vector Laboratories (Burlingame, CA). Anti–5-LO monoclonal antibody was from BD Biosciences (San Diego, CA); rabbit and goat polyclonal anti-AKLOXAP/FLAP antibodies were purchased from Thermo Scientific (Rockford, IL) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Alexa Fluor 488–conjugated donkey anti-mouse and donkey anti-goat and Alexa Fluor 594–conjugated donkey anti-rabbit secondary antibodies were purchased from Life Technologies (Grand Island, NY). N-(25,4R)-(4-(biphenyl-2-ylmethyl)-isobutylamino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl[8-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acylamide (cPLA2 Inh) was obtained from Calbiochem/EMD Biosciences (San Diego, CA). Fura-2 AM was from Life Technologies. The p38 inhibitor SB203580, and the ERK (MEK1 and MEK2) kinase inhibitor U0126 were purchased from Enzo Life Sciences (Farmington, NY) and Cell Signaling Technology (Danvers, MA), respectively.

**Purification and activation of mouse neutrophils**

PMNs were purified from bone marrow of 8- to 12-wk-old mice by density gradient centrifugation with Histopaque 1077 and 1119 (Sigma-Aldrich) as the separation media (Feng et al., 2010). For each experimental condition, 1.0 × 10⁶ PMNs were suspended in 300 μl of DMEM (phenol red free) containing 1.5 mM calcium and magnesium. For priming, PMNs were incubated with 1 ng/ml GM-CSF for 60 min at 37°C in an atmosphere of 5% CO₂; PMNs for control and C5a-only conditions were incubated in media alone for 60 min at 37°C. PMNs were then stimulated with 100 nM C5a for 5 min at 37°C, centrifuged, and the supernatants removed and kept on ice for subsequent LTβ4 analysis. To inhibit cPLA2 activity, cells were incubated with 5 μM cPLA2 Inh for 60 min at 37°C in the presence or absence of GM-CSF, followed by stimulation with C5a for 5 min. To inhibit p38 and ERK MAPK activity, cells were first incubated with 10 μM SB203580 or 10 μM U0126 for 5 min, followed by concomitant incubation of 5 μM cPLA2 Inh and 1 ng/ml GM-CSF for 40 min at 37°C in an atmosphere of 5% CO₂.

Cell pellets were resuspended in 400 μl of 4% paraformaldehyde and transferred to eight-well Millicell EZ slides (EMD Millipore) that were precoated with poly-I-lysine (per manufacturer’s instructions). The cells were allowed to adhere for 10 min, washed with phosphate-buffered saline (PBS), and permeabilized for 5 min at room temperature (RT) with 0.1% Triton X-100 in PBS. The slides were washed in PBS and blocked with 5% fetal bovine serum in PBS for 30 min at RT. Primary antibodies were diluted in blocking solution overnight at 4°C. For 5-LO/FLAP interaction studies, anti–5-LO antibody (1:100) and Thermo Scientific (Waltham, MA) anti-FLAP antibody (1:100) were used; for FLAP domain interaction studies, anti–FLAP N-terminus (1:50; Santa Cruz Biotechnology) and anti–FLAP C-terminus (1:100; Thermo Scientific) were applied. The next day, slides were washed and incubated with Alexa Fluor 488– and Alexa Fluor 594–conjugated secondary antibodies (1:1000 in blocking buffer) for 60 min at RT. Slides were then washed and mounted with Vectashield containing DAPI using a #1.5 glass coverslip. Slides were sealed with nail polish and stored at 4°C until use.
Time-correlated single-photon counting FLIM analysis

Protein interactions were defined by time-correlated single-photon counting FLIM with instrument modifications noted in the following (Mandal et al., 2004, 2008). The baseline lifetimes of Alexa Fluor 488 (donor fluorophore) were calculated by single-exponential-decay fitting of fluorescence emission in the absence of Alexa Fluor 594 (acceptor fluorophore). For samples stained for both donor and acceptor, lifetimes were fit to a biexponential decay with lifetime of one component fixed to the donor-only lifetime. The lifetime for the interacting component, \( \tau_1 \), as well as fractional contributions for the percentage of interacting fluorophores, \( a_1(\% ) \), and the noninteracting component were determined. At least four separate experiments were performed, with the reported n the total number of cells analyzed, and within each cell at least five different pixels were used to determine the mean value.

Intracellular calcium measurements

Purified bone marrow PMNs were counted and loaded with the fluorescent Ca\(^{2+}\) indicator Fura-2 AM (2 μM for 30 min at 37°C) and transferred into a precoated poly-l-lysine 96-well plate. For cells primed with GM-CSF, Fura-2 AM was included during the priming and/or inhibitor incubation periods. Cells were then washed twice with HBSS (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 2 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), 13.8 mM glucose, pH 7.4). The plate was placed on an inverted Zeiss Axiovert 200 microscope (Zeiss, Jena, Germany), and cells were focused using a 40× objective. Cells were then applied to 5 min with C5a, PBS containing 1.5 mM calcium/magnesium for unstimulated controls, or 2 μM ionomycin as a positive control. After excitation at 340 and 380 nm, AxioCamMR and illuminator N HBO 103 were used to collect data. AxioVision software 4.3.0 was used to analyze the data. Data from single cells and cell clusters were normalized, and the change in 340/380 ratio in arbitrary units was documented. Prism, version 5, software (GraphPad, La Jolla, CA) was used to graph data.

Microscopy equipment and settings

Experimental conditions were all performed at room temperature. A Plan APO VC 60× oil DC N2 objective with a numerical aperture of 1.4 was used in all microscopy experiments. A Nikon Ti-E inverted microscope (Nikon, Melville, NY) was used for all epifluorescence and FLIM. Nikon Elements 3.10 imaging software was used to collect epifluorescence data. The filter cube set contained 3 filters: 1) DAPI with excitation range of 340–380 nm, emission range of 435–485 nm, and mirror at 400 nm. 2) Fluorescein isothiocyanate (FITC) with excitation range of 405–495 nm, emission range of 515–555 nm, and mirror at 505 nm. 3) Tetramethylrhodamine isothiocyanate (TRITC) with excitation range of 528–553 nm, emission range of 590–650 nm, and mirror at 565 nm. Brightness and contrast were applied to equally to all images. All images were normalized to equal gamma levels. For FLIM acquisition, Becker and Hickl (Berlin, Germany) BDL-488-SMC Picosecond Diode Laser and both long-pass (HQ500LP) and bandpass (HQ435/50) emission filters were used in combination with a hybrid detector (HPM-100-40 GaAsP Hybrid Detector integrated with a Hamamatsu [Hamamatsu, Japan] R10467-40 hybrid photomultiplier tube). Becker and Hickl SPCM software with DCC was used to acquire FLIM data, and SPCImage 3.0 (Becker and Hickl) software was used for FLIM analysis. Data were imported into Photoshop CS5, version 12.1 x64, InDesign CS5.5, version 7.5, and Illustrator CS5, version 15.1.0 software (Adobe, San Jose, CA), where overall brightness and contrast were adjusted.

Enzyme immunoassay analysis

Cell supernatants were analyzed for LTB\(_4\) with enzyme immunoassay (EIA; Cayman Chemical; Ann Arbor, MI) according to manufacturer’s instructions.

Statistics

For EIA LTB\(_4\) studies, an unpaired two-tailed Student’s t test (±SEM) was performed to obtain p values. For FLIM studies, data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni’s correction for posttest corrections. \( p < 0.05 \) was considered significant.

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