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Specific Leukotriene Receptors Couple to Distinct G Proteins to Effect Stimulation of Alveolar Macrophage Host Defense Functions

Camila M. Peres,*‡ David M. Aronoff,† Carlos H. Serezani,* Nicolas Flamand,* Lucia H. Faccioli,‡ and Marc Peters-Golden‡*

Leukotrienes (LTs) are lipid mediators implicated in asthma and other inflammatory diseases. LTB₄ and LTD₄ also participate in antimicrobial defense by stimulating phagocyte functions via ligation of BLT1 receptor and cysteinyl LT type 1 (cysLT1) receptor, respectively. Although both Goα and Goq proteins have been shown to be coupled to both BLT1 and cysLT1 receptors in transfected cell systems, there is little known about specific G protein subunit coupling to LT receptors, or to other G protein-coupled receptors, in primary cells. In this study we sought to define the role of specific G proteins in pulmonary alveolar macrophage (AM) innate immune responses to LTB₄ and LTD₄. LTB₄ but not LTD₄ reduced cAMP levels in rat AM by a pertussis toxin (PTX)-sensitive mechanism. Enhancement of FcγR-mediated phagocytosis and bacterial killing by LTB₄ was also PTX-sensitive, whereas that induced by LTD₄ was not. LTD₄ and LTB₄ induced Ca²⁺ and intracellular inositol monophosphate accumulation, respectively, highlighting the role of Goαq protein in mediating PTX-insensitive LTD₄ enhancement of phagocytosis and microbicidal activity. Studies with liposome-delivered G protein blocking Abs indicated a dependency on specific Goαq₁₁ and Goα₁₃ subunits, but not Goαq or Goq₇, in LTD₄-enhanced phagocytosis. The selective importance of Goαq₁₁ protein was also demonstrated in LTD₄-enhanced phagocytosis. The present investigation identifies differences in specific G protein subunit coupling to LT receptors in antimicrobial responses and highlights the importance of defining the specific G proteins coupled to heptahelical receptors in primary cells, rather than simply using heterologous expression systems. The Journal of Immunology, 2007, 179: 5454–5461.

The lipid mediators leukotrienes (LT)³ are derived from the 5-lipoxygenase pathway of arachidonic acid metabolism. They are comprised of two specific classes, namely LTB₄ and LTC₄, LTD₄, or LTE₄; the latter are collectively known as cysteinyl LTs (cysLTs). LTB₄ is a potent neutrophil chemoattractant (1), and the cysLTs are best known for their bronchoconstrictive properties, which account for the bioactivity long known as slow reacting substance of anaphylaxis (2). However, both LT classes are now recognized to enhance a variety of leukocyte functions, including adherence, phagocytosis (3), secretion of reactive oxygen intermediates and lysosomal hydrolases (4), as well as synthesis of chemokines (5).

LTB₄ and cysLTs ligate two different types of heptahelical G protein-coupled receptors (GPCRs); these receptors are known as BLT type 1 (BLT1) and type 2 (BLT2) receptors (6, 7) and cysLT type 1 (cysLT1) and type 2 (cysLT2) receptors (8, 9). GPCRs are a large class of receptors that signal though the activation of small heterotrimeric G proteins (Goα, Goβ, and Goγ), as reviewed by Landry et al. (10). These G proteins are classified based on their α subunits into four families: Goαq, which activates adenylyl cyclase and Src tyrosine kinases; Goαq, which classically inhibits adenylyl cyclase but can also activate Src tyrosine kinases; Goαq, which stimulates phospholipase Cβ and Bruton’s tyrosine kinase; and Goα₁₂, which activates Bruton’s tyrosine kinase, GTPase-activating protein 1, and p115 Rho guanine exchange factor Lsc (10). To date, 27 Goα, 5 Goβ, and 14 Goγ subunits have been described, highlighting the complexity of this system (10). Further complicating matters is the fact that many GPCRs can activate more than one G protein family. For example, using recombinant cell system approaches, it was shown that all four LT receptors can couple to both Goαq and Goq proteins, resulting in pertussis toxin (PTX)-sensitive reductions in cAMP and increase in intracellular Ca²⁺, respectively (8, 11). However, the relative role of specific G proteins in signaling from each of these receptors is poorly defined in primary cells.

The alveolar macrophage (AM) serves an important role in innate immune defense in the distal lung, which is essential to preserve pulmonary gas exchange (12). AMs both secrete and respond to both classes of LTs (3, 13, 14). Our laboratory has previously studied the effects of LTs on critical antimicrobial functions of primary AMs, such as the phagocytosis and intracellular killing of...
bacterial pathogens (3, 13, 14). Although LTB₄ and cysLTs each activate antimicrobial functions, there are both similarities and differences in the intracellular signaling mechanisms they use to do so. For example, whereas both LTB₄ and cysLTs are equally potent enhancers of AM phagocytosis of IgG-opsonized bacteria (3), only LTB₄ does so by enhancing the phosphorylation of the tyrosine kinase Syk, a proximal effector in the FcγR signaling pathway (15). Moreover, LTB₄ has been shown to be more potent than cysLTs at stimulating NADPH oxidase activation and bacterial killing in AMs (14). These data indicate that although AMs express receptors for both LT classes, the downstream signal transduction and functional programs they activate are divergent. In this study, we focused on BLT1/cysLT1-elicted responses, as opposed to effects potentially mediated by the BLT2 or cysLT2 receptors. Prior studies demonstrated that 1) BLT1- and cysLT1-selective antagonists fully blocked the effects of LTB₄ and cysLTs (respectively) on phagocytosis (3) and 2) that a dual cysLT1/cysLT2 antagonist showed no additional effects over a cysLT1-selective antagonist in preventing the cysLT-mediated enhancement of bacterial killing in AMs (14).

It is unknown whether these divergent responses reflect differential coupling to distinct classes of G proteins. In the present study, we sought to investigate the role of Goq vs Gqi proteins in mediating the stimulation of AM phagocytosis and bacterial killing induced by LTB₄ and cysLTs.

Materials and Methods

Animals

Pathogen-free 125- to 150-g female Wistar rats were obtained from Charles River Breeding Laboratories and treated according to National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

Reagents

Lipofectin reagent, fura 2-acetoxyethyl ester and pluronidic acid F-127 were obtained from Molecular Probes. PTX was from Calbiochem. LTB₄ and LTD₄ were purchased from Cayman Chemicals. Specific Abs against the following were purchased: phospho-Syk (Tyr531-532) and Syk (Cell Signaling Technology), β-tubulin (Sigma-Aldrich), Gqi (Chemicon International), Goq₁ (DS clone; Biomol), Gqi₃ (T-207-354–354), Goq₁₁ (internal 283–300), and Gq/11 (βγ complex) (Calbiochem). Ionomycin, α-phenylendiamine dihydrochloride, saponin, and peroxidase-labeled rabbit or mouse secondary Abs and ECL Plus detection reagents (Amersham Biosciences).

Isolation and culture of AMs

Resident AMs of >95% purity were obtained from rats via lavage (16) and resuspended in RPMI 1640 at 2 × 10⁶ cells/ml. Cells adhered to tissue culture-treated slides or plates for 1 h (37°C, 5% CO₂) followed by two washes with warm RPMI 1640. Cells were cultured overnight in RPMI 1640 containing 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B before use. The next day cells were washed twice with warm medium to remove nonadherent cells.

FcyR-mediated phagocytosis assays

The phagocytosis of IgG-opsonized, nonviable, FITC-labeled Escherichia coli (Molecular Probes) was assessed as previously reported (17). Adherent cells were treated overnight with PTX (600 ng/ml) (37°C, 5% CO₂) in serum-containing medium. In some experiments, cells were treated overnight with Lipofectin reagent (1% v/v), with or without isotype controls or specific Abs against different G proteins (1/500 dilution) in serum-free medium according to the manufacturer’s instructions and previous reports (18, 19). Some cells were transfected with both Gqi and Goq proteins blocking Abs together (each diluted 1/1000 to maintain the same ratio of total Ab to Lipofectin as when a single Ab was used). The next day, medium was removed and cells were replenished with fresh serum-free medium and allowed to stabilize for at least 1 h before the start of experiments. Drugs of interest were added 10 min before phagocytosis was initiated. Results are expressed as a percentage of the control, to which only vehicle was added.

Tetrazolium dye reduction assay of bacterial killing

The ability of AM to kill Klebsiella pneumoniae bacteria independent of their capacity for phagocytosis was quantitated using a tetrazolium dye reduction assay as described elsewhere (14) and results are expressed as the percentage of survival of ingested bacteria.

Measurement of intracellular cAMP

Macrophages were cultured overnight in 6-well plates in RPMI 1640 plus 10% FBS at a concentration of 3 × 10⁵ cells/well. Medium was then changed to serum-free medium and cells were incubated for 15 min with different concentrations of LTB₄, LTD₄, or vehicle control (DMSO). In some experiments, PTX (600 ng/ml) was added to the cells and incubated for various intervals before adding LTB₄ or LTD₄. Culture supernatants were aspirated and the cells were lysed by incubation for 20 min with 0.1 M HCl (22°C), followed by disruption using a cell scraper. Intracellular cAMP levels were determined by ELISA according to the manufacturer (Assay Designs).

Analysis of calcium mobilization

Freshly isolated AMs were centrifuged for 10 min at 750 × g, and then resuspended in HBSS containing 1.6 mM CaCl₂. The warmed cell suspension (37°C, 5 × 10⁵ cells/ml) was treated with 5 μM fura 2-acetoxyethyl ester in the presence of 30 μg/ml of pluronidic acid F-127 for 1 h to enhance dye solubility and uptake (20). The suspension was washed twice with HBSS containing 1.6 mM CaCl₂, and finally resuspended at a density of 5 × 10⁶ cells/ml and transferred into the magnetically stirred cuvette of a luminescence spectrometer (LSSOB; PerkinElmer). Calcium mobilization was monitored using excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Data are presented as the ratio of fluorescence obtained at 340 and 380 (340/380) nm.

Measurement of intracellular IP₁ accumulation

Macrophages were cultured overnight in 24-well plates in RPMI 1640 plus 10% FBS at a concentration of 2 × 10⁵ cells/well. Medium was then changed to serum-free medium and cells were incubated for 1 h with 100 nM LTB₄, LTD₄, or vehicle control. Culture supernatants were aspirated and the cells were lysed by incubation for 30 min with 2.5% of lysis reagent (37°C). Intracellular IP₁ levels were determined by ELISA according to the manufacturer.

Immunoprecipitation and Western blot analysis

Immunoprecipitation of Syk protein from AM lysates was performed as we have previously published (15). Western blotting was performed as previously described (21). Protein samples (40 μg) were resolved on 8% Tris-HCl polyacrylamide gels (containing 6 M urea for Gqi subunit Western blots) and subsequently transferred to a nitrocellulose membrane. Membranes were probed with primary Abs followed by HRP-conjugated anti-rabbit or anti-mouse secondary Abs and ECL Plus detection reagents (Amersham Biosciences).

Statistical analysis

Data are presented as the mean ± SEM and were analyzed with the Prism 4.0 statistical program (GraphPad Software). Comparisons among three or more experimental groups were performed with ANOVA followed by the Bonferroni correction. Differences were considered significant for values of p < 0.05. Experiments were performed on three or more separate occasions unless otherwise specified.

Results

LTB₄ but not LTD₄ decreases intracellular cAMP in AMs

Both BLT1- and cysLT1-transfected cells exhibit dual coupling to Gqi and Goq proteins (7, 22, 23); however, few data are available in primary cells. To determine the relative contribution of Gqi protein in BLT1 and cysLT1 signaling, our first approach was to investigate the levels of intracellular cAMP in rat AMs stimulated with LTB₄ or LTD₄ (Fig. 1). Rat AMs treated with LTB₄ (15 min) showed a concentration-dependent decrease in intracellular cAMP. Decreases were observed at LTB₄ concentrations as low as 100
Intracellular cAMP concentrations were determined as described in Materials and Methods. Data are expressed as relative units with the control value to which only vehicle was added = 1. Data are mean ± SEM for 3–10 separate experiments. *p < 0.05 compared with control.

**FIGURE 1.** Effect of LTB4 and LTD4 on intracellular cAMP concentration ([cAMP]i) in AM. AMs were incubated for 15 min with or without LTB4 or LTD4 at the indicated concentrations. Intracellular cAMP concentrations were determined as described in Materials and Methods. Data are expressed as relative units with the control value to which only vehicle was added = 1. Data are mean ± SEM for 3–10 separate experiments. *p < 0.05 compared with control.

The data suggest that LTB4, but not LTD4, activates a G protein coupled receptor.

**AMs express Gαi subunits 2 and 3 and Gαq/11.** Members of the Gα family include Gα11, Gα12, and Gα13 subunits and of the Gαq family include Gαq11, Gαq12, Gαq14, Gαq15, and Gαq16 (10). Previous studies of G protein expression in AMs did not discriminate clearly among the three Gαi subunits (24–26). To address that perspective, the expression of all three Gαi subunits and Gαq/11 in rat AM was examined by Western blot. Fig. 2A demonstrates the same membrane stripped and re-immunoblotted with Abs to the various G proteins. We observed bands at ~42 kDa corresponding to Gαi2 and Gαi3, and the characteristic doublet at 42 and 43 kDa representative of Gαq11 and Gαq14, respectively. However, Gαq11 expression was not detected by this method. As a positive control for the Gαq11 Ab, we used rat brain and cerebellar cell lysates that are well known to express Gαq11 protein (27). Unlike AMs, a band at the expected molecular mass for the G protein (~42 kDa) was observed in both cultured cell types (Fig. 2B).

We next used PTX in pharmacological studies of LT signaling in AMs. PTX is a classical inhibitor of Gα proteins that works by covalently ADP-ribosylating their α subunits, uncoupling them from their cognate membrane-bound receptors and thus preventing their activation (10). To evaluate the state of ADP ribosylation of Gαi2 and Gαi3 subunits in PTX-treated cells, we performed immunoblot analyses under conditions optimal for the separation of ribosylated subunits from their nonribosylated forms (28). This method allows the identification of a characteristic ~2-kDa upward shift in apparent molecular mass that occurs after ADP-ribosylation (28). As shown in Fig. 2C, ADP ribosylation was clearly seen in both Gαi2 and Gαi3 after 3 and 18 h incubation with active PTX (600 ng/ml), but not with heat-inactivated PTX. Slight ADP ribosylation of Gαi3 was observed as early as 30 min. As all AM Gαq subunits were completely ribosylated after overnight treatment, this time point and PTX dose were used in additional experiments.

**PTX treatment prevents reduction of cAMP by LTB4.** We next used PTX to explore the participation of Gαi proteins in mediating the LTB4-induced decrease in intracellular cAMP release. AMs were pretreated with PTX overnight, followed by addition of LTB4 (1 nM) for 15 min. We found that PTX treatment completely abrogated the inhibitory effect of LTB4 on intracellular cAMP levels (Fig. 3A).

**PTX differentially modulates enhancement of FcγR-mediated phagocytosis by LTB4 and LTD4.** Our laboratory has demonstrated that FcγR-mediated phagocytosis by AMs is enhanced by LTB4 and cysLTs (3, 29), though the exact mechanisms involved remain unclear. We hypothesized, based on our findings, that LTB4 stimulation of FcγR-mediated phagocytosis would be at least partially PTX-sensitive, whereas the effects of LTD4 would not. Concentrations of 1 and 100 nM, respectively, of LTB4 and LTD4 were used because 1) those concentrations effectively enhanced both phagocytosis and bacterial killing in our previous studies (3, 14) and 2) they resulted in a maximal decrease (LTB4) and no effect (LTD4) in cAMP (Fig. 1). As expected, LTB4 and LTD4 increased the phagocytosis of IgG-E. coli in AMs by 50.0 ± 3.4% and 37.2 ± 8.5%, respectively (Fig. 3B). However, pretreatment of the cells with PTX completely abolished the ability of LTB4 to enhance FcγR-mediated ingestion, but did not significantly attenuate LTD4 enhancement (Fig. 3B). These data suggest that stimulatory effects of BLT1 engagement on AM phagocytosis follow its ability to acutely decrease cAMP via Gαq activation, whereas those of cysLT1 ligation are PTX-insensitive.

**LTB4-enhanced Syk activation is blocked by PTX.** We previously showed in AMs that LTB4, but not other 5-lipoxygenase products including LTD4, increased phosphorylation of the protein tyrosine kinase Syk, a proximal effector in the FcγR signaling pathway (15). We postulated that PTX would likewise suppress Syk phosphorylation following LTB4 treatment in AMs. As previously found at 100-fold higher concentrations (15), the addition of 1 nM LTB4 3 min before IgG-SRBC challenge augmented the degree of Syk phosphorylation evoked by FcγR engagement (Fig. 3C). Indeed, PTX treatment attenuated the ability of LTB4 to enhance Syk phosphorylation (Fig. 3C) by ~38% as shown by
PTX treatment prevents reduction of cAMP by LTB4 and differentially modulates AM innate immune responses to LTB4 and LTD4. Cells were pretreated with PTX for 18 h, followed by LTB4 treatment at a final concentration of 1 nM for 15 min. Intracellular cAMP concentrations ([cAMP]) were determined as shown in Materials and Methods. Data are expressed relative to the appropriate control value to which PTX was or was not added. Data are mean ± SEM from three separate experiments. B. AMs were preincubated with PTX for 18 h. The next day, cells were incubated with LTB4 (1 nM) or LTD4 (100 nM), or vehicle control for 10 min and then challenged with E. coli opsonized with specific rabbit polyclonal IgG. Phagocytic index (PI) was calculated as described (17) and expressed as a percentage of the control value in which no drugs were added. Mean ± SEM data are shown from four to seven separate experiments, each performed in sextuplet. C. Cells were pretreated for 18 h with PTX or vehicle and then incubated with 1 nM LTB4 for 3 min before the addition of IgG-SRBC (1:33 ratio), followed by incubation for 7 min at 37°C. Incubations were terminated by the addition of lysis buffer, and lysates were subjected to immunoprecipitation and immunoblotting. Immunoblots represent phosphorylated Syk detected with anti-phospho-Syk Ab (top) and the total amount of Syk protein evaluated with an anti-Syk Ab (bottom). The densitometry analysis represents mean ± SEM from independent experiments (right). D. AMs were preincubated with PTX and the next day were infected with 50:1 opsonized K. pneumoniae. Thirty min after infection, the cells were incubated with LTB4 (1 nM), LTD4 (100 nM), or vehicle. Microbicidal activity was assessed and expressed as the mean ± SEM percentage survival of ingested bacteria from four independent experiments, each performed in triplicate. *, p < 0.05; **, p < 0.001; *** p < 0.001 only vs control; #, p < 0.001 vs LTB4-treated cells.

PTX differentially modulates enhancement of bacterial killing by LTB4 and LTD4

Along with phagocytosis, microbicidal activity is a critical step in the control of infection. We have demonstrated that endogenous and exogenous LTs enhance bacterial killing (14). We therefore determined the importance of Gαq proteins in coupling BLT1 and cysLT1 to effects on killing of the clinically relevant Gram-negative pathogen K. pneumoniae. As previously demonstrated (14) and in Fig. 3D, both exogenous LTB4 (1 nM) and LTD4 (100 nM) increased AM microbicidal activity, reflected by a decrease in survival of ingested bacteria (~29% and 38%, respectively). Cell pretreatment with PTX abolished the LTB4 effects on bacterial killing (Fig. 3D), but not those of LTD4. These results indicate that although LTB4 and LTD4 are both capable of enhancing AM bacterial activity, only the former does so via coupling of its cognate receptor to Gαq.

LTB4 and LTD4 activate phospholipase C (PLC)β

Given that LTD4 potentiation of FcγR-mediated phagocytosis and microbicidal activity in AM was PTX-resistant (Fig. 3, B and D), and in light of the fact that the Gαq pathway appears to be important in mediating cysLT receptor signaling in transfected cells (8, 11, 30), we examined Gαq-mediated signaling by LTB4 and LTD4 in AM by evaluating Ca2+ mobilization and IP1 accumulation (IP1 is a stable product of inositol 1,4,5 triphosphate derived from PLCβ activation). As shown in Fig. 4A, 300 nM LTD4 potently induced intracellular Ca2+ elevation. This response to LTD4 was totally blocked when cells were pretreated for 20 min with the cysLT1 antagonist, MK 571 (1 μM). Unlike LTD4, LTB4 was unable to mobilize intracellular Ca2+ to a detectable degree over a range of concentrations (1–300 nM) (Fig. 4B). This result was surprising because we have previously demonstrated that LTB4 increased intracellular Ca2+ concentration in murine dendritic cells (31) and in human neutrophils (32). We therefore used an additional approach for assessing Gαq activation, namely, an ELISA to quantitate intracellular IP1 accumulation. Although LTB4 failed to induce Ca2+ mobilization, it (100 nM) did significantly increase accumulation of IP1 in a manner similar to LTD4 (Fig. 4C).

Ab blockade indicates that LTB4 responses are dependent on Gαq and Gαq subunits, whereas LTD4 responses are mediated by Gαq

As an alternative approach to assess the roles of Gαq and Gαq in mediating the effects of LTs in AMs, and in an effort to discern
roles of Gβγ and specific Ga isoforms, phagocytosis studies were performed in cells pretreated overnight with Abs raised against rat Ga12, Ga13, Gaq11, and Gβγ proteins or isotype controls (namely, IgG2b, the isotype control for anti-Ga12 or IgG, the isotype control for anti-Ga13 and anti-Ga1). Abs were complexed with liposomes to facilitate delivery into the cytosol (18, 19). As expected, 1 nM LTB4 and 100 nM LTD4 significantly increased FcγR phagocytosis compared with vehicle-treated cells (41 ± 4.8% and 94.6 ± 31%, respectively) (Fig. 5). Although Ga12 and Ga13 were expressed in AM, treatment of the cells with Abs against Ga13 completely abolished the ability of LTB4 to enhance phagocytosis, whereas anti-Ga12 Abs had no effect (Fig. 5A). When anti-Ga12 and anti-Ga13 were used together, phagocytic activity fell slightly below that of untreated cells. To confirm the lack of Ga13 involvement, we conducted additional experimental controls. We found that the responses to isotype control IgG2b and anti-Ga13 in the presence of LTB4 were similar, and that the responses to anti-Ga12 plus anti-Ga13 and isotype control IgG2b plus anti-Ga13, although slightly different, were not statistically significant, suggesting no role for Ga13 (Fig. 5A). AMs were also transfected with Lipofectin with Gβ protein blocking Abs and/or the isotype control Abs in the absence of LT before assaying phagocytosis. No alterations in basal phagocytic capacities were observed with any of the blocking and isotype control Abs (data not shown), suggesting that this procedure does not cause a nonspecific depression of phagocytosis, instead suggesting that the effects of the blocking Abs are indeed specific to stimulation by LT. Consistent with the enhancement of LTB4- and LTD4-mediated IP1 and Ca2+ signaling, the blockade of Gaq11 proteins, whereas the former also used the Ga13 protein.

Discussion
In the present study, we extended our exploration of the mechanisms by which LTB4 and LTD4 modulate AM phagocytosis and bactericidal activity by focusing on the coupling of BLT1 and cysLT1 receptors to specific Ga proteins. Our results demonstrate that 1) LTB4, but not LTD4, decreases intracellular cAMP levels, a phenomenon prevented by PTX; 2) although FcγR-mediated phagocytosis and microbicidal activity are enhanced by both LTs, only LTB4 does so in a PTX-sensitive manner; 3) both LTD4 and LTB4 elicit PLCβ activation, as indicated by the ability of LTD4 to evoke detectable increases in intracellular Ca2+ and of both LTs to increase IP1; and 4) the blockade of specific Ga proteins indicates that whereas Ga13 and Gaq proteins are used for LTB4-enhanced...
phagocytosis, only $G_{i3}$ is used by LTD$_4$. A schematic representation of the role of $G_{i1}$ and $G_{i3}$ subunits in AM responses to LTs is depicted in Fig. 6.

Previous studies using transfected cell lines have shown that BLT1 and cysLT1 are coupled to both $G_{i1}$ and $G_{i3}$ proteins (7, 8, 11, 22, 23), with the relative importance of these G proteins varying depending on the cell type and functions under investigation. For example, in BLT1-expressing RBL cells LTB$_4$-induced chemotaxis, but not phosphatidylinositol hydrolysis and calcium mobilization, depended on $G_{i1}$ proteins (33). Hoshino et al. (34) demonstrated in human monocyte-like THP-1 cells that LTD$_4$ signals through PTX-sensitive and PTX-insensitive pathways to mediate cellular activation. In human neutrophils, it has been shown that LTB$_4$-induced calcium mobilization, chemotactic migration, and degranulation were PTX-sensitive, suggesting coupling to $G_{i}$ protein (32). However, there is no information regarding the relative importance of particular G protein classes or subunits in LT receptor-mediated enhancement of phagocytosis and bacterial killing. In fact, we know of no such information about specific G protein subunit coupling to LT receptors in primary cells. In view of the importance of the AM in antimicrobial defense of the lung and as a target for the immunostimulatory actions of LTs, it is a relevant cell type in which to focus this investigation.

To address the participation of $G_{i1}$ protein in LTB$_4$- and LTD$_4$-mediated responses, we assessed the effects of each LT on intracellular cAMP levels. A clear distinction between the two was observed, as low concentrations of LTB$_4$ but not LTD$_4$ were able to reduce intracellular cAMP. LTB$_4$ was very potent in this regard, as picomolar concentrations of LTB$_4$ suppressed cAMP generation, and the effect was totally abolished by PTX, implicating a role for $G_{i1}$ signaling. These results are in agreement with other studies that have found that LTB$_4$ inhibited forskolin-activated cAMP production by Chinese hamster ovary cells expressing BLT1 (7, 35). In contrast, LTB$_4$ was found to increase cAMP in human neutrophils (36, 37). Such data highlight the underappreciated complexity of GPCR signaling in unique cell types. Unlike LTB$_4$, cysLTs were reported not to alter cAMP levels in leukocytes (37) or in human airway smooth muscle cells (38), but to increase cAMP in a human epithelial cell line (39).

The suppressive effects of PGE$_2$, a lipid mediator derived from the cyclooxygenase pathway, on AM phagocytosis and bacterial killing follow its ability to elevate cAMP via $G_{i}$-coupled receptors (17, 40). Considering that $G_{i}$ protein activation serves as a negative regulator of $G_{i}$ signaling (41), we hypothesized that changes in intracellular levels of cAMP might contribute to the opposing effects of LTs and PGE$_2$ on AM innate immune responses.

Although both LTB$_4$ and cysLTs enhance FcγR-mediated phagocytosis and bacterial killing, we previously identified important differences in the molecular mechanisms triggered by BLT1 and cysLT1 activation that are responsible for these effects (14, 15). For example, LTB$_4$, but not cysLTs, amplifies FcγR-induced Syk activation through a Ca$^{2+}$-dependent mechanism (15). We now demonstrate that BLT1 activation, but not cysLT1 activation, enhances FcγR-mediated phagocytosis and Syk phosphorylation in a PTX-sensitive manner (Fig. 3, B and C), revealing a role for $G_{i1}$ proteins that was to our knowledge undocumented. Recently, Kuniyeda et al. (42) identified, in transfected cells, the intracellular region of BLT1 responsible for specific G$_{i3}$-protein coupling.

Previous work by other groups established that murine peritoneal macrophages express $G_{i1}$ and/or $G_{i3}$ as well as $G_{i4}$ (26), and guinea pig AMs express $G_{i4}$ and/or $G_{i3}$ but not $G_{i1}$ (24). However, these studies were limited by the use of reagents that could not discriminate between $G_{i1}$ and $G_{i3}$. Using more specific Abs, we demonstrated that only $G_{i3}$, not $G_{i1}$, is expressed in primary rat AMs, along with both $G_{i3}$ and $G_{i4}$. Moreover, although both $G_{i3}$ and $G_{i4}$ were ribosylated by PTX treatment, we were able to determine the relative importance of individual $G_{i}$ subunits in mediating coupling to BLT1 and cysLT1 by using Abs raised against rat $G_{i2}$, $G_{i3}$, $G_{i4}$. To facilitate their entrance into the cells, Abs were complexed with liposomes, taking advantage of a novel intracellular delivery system that was developed in cell lines (18, 19). We believe this report is the first published using this functional knockdown approach in a primary cell. Other approaches have been used to block specific G proteins, including genetic deletion (43) and small interfering RNA technology (44). We chose to use the Lipofectin-based strategy for several reasons. First, primary AMs are difficult to transfec with small interfering RNA (45) and small interfering RNA has been shown to trigger antiviral responses that might significantly alter immune responses in vitro (46). Importantly, we did not find significant effects of liposome treatment on AM phagocytosis. Second, genetic knockdown can be complicated by compensatory alterations in expression of nontargeted G proteins. Lastly, liposomes are easy to use and the approach provides a facile method for protein blockade that is limited mostly by the availability of blocking Abs. The results of these studies (Fig. 5) indicated that LTB$_4$/BLT1 effects were mediated by $G_{i3}$ and $G_{i4}$ whereas LTD$_4$/cysLT1 effects were mediated solely by $G_{i3}$. Oh and Schnitzer (47) showed that $G_{i3}$ preferentially localizes in caveolae, while $G_{i3}$ and $G_{i4}$ localize in lipid rafts, suggesting compartmentation of GPCR signaling proteins in specific membrane microenvironments as a potential determinant of receptor-effector coupling.

The $G_{i}/G_{i1}/G_{i3}/G_{i4}$ family is coupled to certain receptors and activates PLCβ subtypes to mediate phosphatidylinositol hydrolysis, resulting in Ca$^{2+}$ elevation (48). To address the activation of $G_{i}/G_{i1}$, we first evaluated Ca$^{2+}$ mobilization in response to...
LTB4 and LTD4. Our data show that LTD4 (300 nM) elevated Ca2+², whereas LTB4 (300 nM) did not. This result was unexpected because a rise in cytosolic Ca2+² concentration upon LT B4 ligation of BLT1 is widely recognized in neutrophils (49). These inconsistent data are also underscored by the requirement for intracellular Ca2+² in Syk activation by LTB4 during the ingestion of IgG-opsonized targets (15). In light of this requirement, we used an additional approach for assessing Goq activation, namely, inositol 1,4,5 triphosphate release in response to PLCβ activation, as measured by an ELISA for intracellular IP1 production. Although LTB4 failed to induce Ca2+² mobilization, it did increase accumulation of IP1 in a manner similar to LTD4, suggesting that LTB4 indeed activates a Goq-coupled receptor. It is possible that the IP1 assay may be more sensitive than Ca2+² mobilization in our experimental system.

Although previous studies have established the role of specific G proteins in mediating signaling from LT receptors and other GPCRs in cell lines or transfected cells, our study explores this interaction as regards innate immune functions of rat primary macrophages. However, the relevance of our findings for other primary cell types is still uncertain and will require direct examination. Our data reveal a major divergence in the role of individual G protein subunits in mediating the stimulation of AM phagocytosis and microbicidal activity by LTB4 and LTD4. BLT1 uses both Goq13 and Goq4 proteins to activate these responses, whereas cysLT1 does so only via Goq4. These findings emphasize the need to dissect signaling programs activated by specific GPCRs in relevant primary cells.

Disclosures
The authors have no financial conflict of interest.

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