Leukotriene B₄ Potentiates CpG Signaling for Enhanced Cytokine Secretion by Human Leukocytes

Éric Gaudreault and Jean Gosselin

*J Immunol* 2009; 183:2650-2658; Prepublished online 20 July 2009;
doi: 10.4049/jimmunol.0804135
http://www.jimmunol.org/content/183/4/2650

---

**References**  This article cites 56 articles, 33 of which you can access for free at:  
http://www.jimmunol.org/content/183/4/2650.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Leukotriene B₄ Potentiates CpG Signaling for Enhanced Cytokine Secretion by Human Leukocytes

Éric Gaudreault and Jean Gosselin

TLRs are known to be important in innate host defense against a variety of microbial infections. In particular, TLR9 has been associated with immune defense against different foreign organisms by recognition of unmethylated DNA sequences. In this report, we provide evidence that leukotriene B₄ (LTB₄) has the capacity to modulate TLR9 expression on human neutrophils. The effect of LTB₄ was found to be specific, because related leukotrienes such as LTC₄ and LTD₄ or neutrophil agonists IL-8 and C5a failed to modulate TLR9 expression in neutrophils. Using fluorochrome-tagged CpG DNA, we observed that LTB₄ treatment also increased TLR9 ligand binding in neutrophils. Moreover, LTB₄ stimulation potentiated CpG-mediated signaling via an endosome-independent mechanism in human neutrophils, leading to enhanced secretion of proinflammatory cytokines. The increase in cytokine secretion by LTB₄ following CpG stimulation of neutrophils was associated with the activation of TGF-β-activated kinase (TAK-1) as well as p38 and c-Jun (JNK) kinases. In contrast, in PBMC LTB₄ leads to an increase in cytokine secretion following monocytes (13), as well as in plasmacytoid dendritic cells (14).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Abbreviations used in this paper: ODN, oligodeoxynucleotide; DA, DNA-dependent activator of IFN regulatory factors; LT, leukotriene; TAK-1, TGF-β-activated kinase-1; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; WT, wild type; PBMC, peripheral blood mononuclear cell.

Following DNA entry into the cells and binding to TLR9 in endosomal compartments, recruitment of the adaptor protein MyD88 to the Toll/IL-1R (TIR) domain of the receptor is triggered followed by the engagement of other adaptor proteins of the IL-1R-associated kinase (IRAK) family and of TNFR-associated factor-6 (TRAF6) (4). The engagement of TLR9 leads to the activation of two important but distinct intracellular signaling pathways involving, on the one side, the MAPK family such as p38, JNK, and ERK 1/2 kinases, and on the other side, IκB, resulting in respective activation of the transcription factors AP-1 and NF-κB (reviewed in Ref. 15). During the last few years, a growing body of evidence has shown that TLR9-independent cell activation by DNA was possible. For example, bacterial DNA is known to activate human neutrophils independently of TLR9 engagement, leading to CD11b cell surface modulation (16). Transfection of dsDNA in macrophages and dendritic cells can also lead to the secretion of TNF-α independently of TLR9 activation (17, 18). It was recently reported that CpG-ODN stimulation can lead to the activation of members of the Src family kinases Hck and Lyn as well as the adhesion and migration of THP-1 monocytes and human monocyte-derived dendritic cells in a chloroquine-insensitive manner (19).

Leukotriene (LT) B₄ (LTB₄) is an eicosanoid derived from cell membrane arachidonic acid under the action of the enzymes 5-lipoxygenase and LTA₄ hydrolase. LTB₄ proinflammatory actions on human leukocytes have been well characterized, particularly in neutrophils. These actions include neutrophil chemotaxis, cytokine and chemokine production, secretion of granules, phagocytosis, and induction of the oxidative burst. Complementary to its prominent role in inflammation, evidence has been gathered and has linked LTB₄ to antimicrobial immunity. In fact, we observed that LTB₄ exerts very interesting antiviral properties against different viruses, including human and murine CMV and influenza virus (20–22). We also observed that LTB₄ administration can lead to the secretion of anti-HIV factors, particularly the antimicrobial peptides of the defensin-α family as well as the chemokine MIP-1β (23).
In this report, we sought to determine the potential role of LTB₄ on the expression and signaling of intracellular TLRs in human leukocytes. We demonstrate that LTB₄ has the capacity to modulate TLR9 expression on human neutrophils and to lead to a potentiation in CpG-mediated cytokine release by both PBMC and neutrophils.

Materials and Methods

**Human leukocyte isolation**

Human leukocytes were obtained from the peripheral blood of healthy, medication-free volunteers after informed consent in accordance with an Internal Review Board-approved protocol. Briefly, PBL were enriched by dextran sedimentation followed by centrifugation over a lymphocyte separation medium cushion at 1400 rpm for 20 min. Peripheral blood mononuclear cells (PBMC) were collected at the interface, whereas neutrophils were obtained from the pellet as previously described (24). Neutrophil preparations were depleted of erythrocytes by osmotic shock and then washed and resuspended in HBSS buffer before use. Purity of neutrophil preparations was >99%. No significant percentage of other types of granulocyte was detected in the cell preparation as evaluated by cytometry analysis.

**Mice**

C57BL/6 mice (Charles Rivers Laboratories), as well as MyD88⁻/⁻ and TRIF⁻/⁻ (where TRIF is Toll/IL-1R domain-containing adapter inducing IFN-β) mice (provided Dr. S. Rivest, Université Laval, Québec, Canada and Dr. T.-j. Lin, Dalhousie University, Halifax, Nova Scotia, Canada, respectively), were used during this study and kept in a pathogen-free animal facility. Splenocytes were isolated and stimulated ex vivo with LTB₄, CpG 1826 (Alpha DNA), or a combination of stimuli for 6 h. Following stimulation, cell-free supernatants were assayed for murine TNF-α as described below. LTB₄ was obtained as an ethanolic solution of the acid form and prepared by dilution of the ethanolic LTB₄ in a saline solution containing 0.45% (w/v) NaCl and 0.25% (w/v) dextrose. A saline solution without the addition of LTB₄ was used as a placebo.

**CpG binding assay**

For a binding assay, human neutrophils (2 x 10⁶ cells) were incubated or not with LTB₄ (100 nM) for 30 min in the absence or presence of inhibitory ODN sequences (100 μg/ml) (Alpha DNA). Cyanine-3-tagged CpG-ODN 2216 (Alpha DNA) at the indicated concentrations was then added to the cell cultures and binding was allowed at 37°C for 4 h. Cells were then washed a minimum of three times with HBSS and cyanine-3 expression was analyzed by flow cytometry on 10,000 cells per sample using an EPICS XL apparatus (Beckman Coulter).

**RT-PCR analysis**

Neutrophils (10 x 10⁶ cells) were stimulated with or without LTB₄ (100 and 1000 nM) for 2 h. Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. DNase-treated RNA (1 μg) was reverse transcribed using SuperScript reverse transcriptase (Invitrogen), and PCR was performed using the following specific primers: TLR3, 5'-ATGGGCTTTGAGAAACATTCTCTTC-3' (forward) and 5'-GTTAGATTTAAACATTCCTCTTCG-3' (reverse); TLR7, 5'-TTACCTGGATGGAAACCAGCTACT-3' (forward) and 5'-GTTACCTGGATGGAAACCAGCTACT-3' (reverse); TLR8, 5'-AATCTTCCTTTTCGTTGTCATCC-3' (forward) and 5'-GGTGTAGTCCGCAAGCTTCTGTC-3' (reverse); TLR9, 5'-GGAGAACTTGCAGGCTTTGTGTC-3' (forward) and 5'-GGAGAACTTGCAGGCTTTGTGTC-3' (reverse); GAPDH, 5'-CCCACTATGGCCAAATTTCCATGGCA-3' (forward) and 5'-CCCACTATGGCCAAATTTCCATGGCA-3' (reverse). Amplified DNA was run on 1% agarose gel and stained with ethidium bromide. DNA was visualized under UV light.

**ELISA**

Purified human neutrophils, PBMC, and murine splenocytes (5 x 10⁶ cells) were pretreated or not with the appropriate pharmacological inhibitors of the p38 kinase (SB203580) (Invivogen) and ERK 1/2 (PD98059), P38K (LY294002), and JNK (SP600125) (Sigma Aldrich) for 15–60 min before stimulation with or without LTB₄, CpG-ODN 2216 (human cells), 1826 (murine cells), or a combination of stimuli for the indicated times.

Flow cytometry analysis

Human neutrophils (2 x 10⁶ cells) were stimulated with or without 100 nM LTB₄, LTC₄, or LTD₄ (provided by Dr. M. Rola-Pleszczynski, Université de Sherbrooke, Sherbrooke, Quebec, Canada) or IL-8 or C5a (provided by Dr. M. Pouliot, Université Laval, Quebec, Quebec, Canada) for the indicated times. Cells were fixed for 20 min at room temperature with 2% paraformaldehyde and permeabilized for 2 min in methanol on ice. Cells were then incubated in the presence of anti-FITC-conjugated TLR9 Abs (HyCult Biotechnology) for 30 min. Intracellular TLR9 expression was analyzed on 10,000 cells per sample using an EPICS XL apparatus (Beckman Coulter).

**Western blot analysis**

Human neutrophils or PBMC (5 x 10⁶ cells) were stimulated or not with LTB₄, CpG-ODN 2216, or a combination of stimuli for the indicated time periods. Where indicated, cells were pretreated or not with chloroquine (Sigma-Aldrich) for 30 min before stimulation. Total proteins were extracted in lysis buffer (Sigma-Aldrich) and subjected to electrophoresis on 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane and blocked for 2 h with 5% dry milk diluted in Trition-Tris (TTBS) buffer at room temperature. Membranes were then incubated overnight with primary anti-phospho-p38, anti-phospho-TAK-1 (where TAK-1 is TGF-β-activated kinase-1), anti-phospho-JNK, or anti-p38 Abs (Cell Signaling Technology) at 4°C. Incubation with HRP-conjugated secondary Ab was then performed for 1 h at room temperature and protein bands were visualized by ECL (PerkinElmer).

**Statistical analyses**

Data were analyzed by one-tailed ANOVA followed by a Newman-Keuls posthoc test using PRISM3 software. Differences were considered significant at p < 0.05.

**Results**

**LTB₄-mediated TLR9 up-regulation in human neutrophils**

We previously demonstrated that LTB₄ stimulation of human neutrophils led to an up-regulated expression of cell surface TLR2. In this report, we first sought to determine whether LTB₄ could also modulate intracellular TLR expression in human neutrophils. Neutrophils were therefore stimulated with LTB₄ for 2 h and TLR3, TLR7, TLR8, and TLR9 mRNA levels were determined by RT-PCR. As shown in Fig. 1A, stimulation with two different physiological doses of LTB₄ led to an up-regulation in mRNA levels for TLR7, TLR8, and TLR9. As expected, no TLR3 mRNA signal could be detected in human neutrophils in the absence or presence of LTB₄ in accordance with published data (11). TLR9 is known to be critical for viral recognition of herpes viruses (25–28). Moreover, we previously reported antiviral LTB₄ properties against human and murine CMV and HSV-1 (20, 22). For those reasons, we next verified the implication of LTB₄ in the modulation of TLR9 expression on human neutrophils. Neutrophils were stimulated with LTB₄ for 15, 30, 60, and 120 min and expression levels of TLR9 were then assessed by flow cytometry analysis using anti-TLR9 mAbs. As shown in Fig. 1B, LTB₄ stimulation of human neutrophils resulted in a fast and transitory up-regulation in intracellular TLR9 expression, peaking at 15 min poststimulation. TLR9 expression returned to the basal level after 2 h of stimulation with LTB₄. Because expression of the TLR9 protein does not correlate with mRNA levels, we believe that such a fast modulation of the TLR9 protein we observed should be related to the trafficking of the preassembled TLR9 from endoplasmic reticulum to endosomes following cell stimulation, as demonstrated previously (29). Taken together, these results demonstrate that LTB₄ stimulation of human neutrophils leads to the modulation in intracellular TLR expression, particularly that of TLR9. To rule out the possibility that LTB₄ might activate neutrophils in a nonspecific manner leading to TLR9 modulation, neutrophils were pretreated with the specific BLT1 antagonist U75302 (10 μM) before LTB₄ stimulation. Although LTB₄ led to enhanced TLR9 expression in neutrophils,
no modulation in TLR9 expression was observed in cells pretreated with U75302 before LTB4 stimulation (Fig. 1C). This result demonstrates a specific LTB4 effect on TLR9 expression in human neutrophils. We next investigated whether TLR9 modulation in neutrophils induced by LTB4 is solely a consequence of general cell activation. For that purpose, neutrophils were stimulated with LTB4 or with related LTs, LTC4, and LTD4 (Fig. 1D).

In fact, neutrophils have been reported to express low levels of LTC4 and LTD4 receptors and are slightly responsive to such ligands (30, 31). Specific neutrophil ligands were also used including IL-8 and C5a (Fig. 1E). Although LTB4 led to modulation in TLR9 expression, LTC4, LTD4, IL-8, and C5a failed to positively modulate TLR9 expression in human neutrophils. This last set of experiments strongly supports a specific role for LTB4 in

FIGURE 1. LTB4 modulates intracellular TLR expression in human neutrophils. A, Human neutrophils (10 x 10^6 cells) were stimulated with or without LTB4 (100 and 1000 nM) for the indicated times. Following stimulation, total RNA was extracted and RT-PCR was performed using specific primers for human TLR3, TLR7, TLR8, and TLR9. GAPDH was used as an internal control. Each blot is representative of a minimum of three independent donors tested. The histogram represents densitometric analysis performed on a minimum of three donors tested; *, p < 0.05. B, Human neutrophils (2 x 10^6 cells) were left untreated (gray histogram) or stimulated with LTB4 (100 nM) for 15, 30, 60, or 120 min. Cells were then fixed in paraformaldehyde, permeabilized with methanol, and TLR9 expression was assessed by flow cytometry. The histograms are representative of three independent donors tested. The dotted line represents cell autofluorescence. C, Human neutrophils (2 x 10^6 cells) were preincubated or not with U75302 (10 nM) for 1 h. Following incubation with the antagonist, cells were left untreated (gray histogram) or stimulated with LTB4 (100 nM) for 1 h. Cells were then fixed and TLR9 expression was assessed by flow cytometry. Histograms are one representative of three independent donors tested. Dotted line represents cell autofluorescence. D, Human neutrophils (2 x 10^6 cells) were left untreated (gray histogram) or stimulated with LTB4 (100 nM), LTC4 (100 nM), or LTD4 (100 nM) for 1 h. Cells were then fixed and TLR9 expression was assessed by flow cytometry. Histograms are representative of three independent donors tested. Dotted line represents cell autofluorescence. E, Human neutrophils (2 x 10^6 cells) were left untreated (gray histogram) or stimulated with LTB4 (100 nM), IL-8 (100 nM), or C5a (100 nM) for 1 h. Cells were then fixed and TLR9 expression was assessed by flow cytometry. Histograms are one representative of three independent donors tested. Dotted line represents cell autofluorescence. F, Human neutrophils (2 x 10^6 cells) were preincubated with (dotted line) or without LTB4 (solid line) for 1 h. Following preincubation, a binding assay was performed with cyanine-3-tagged CpG 2216 (1, 3, and 5 μg/ml) for 4 h. CpG binding was assessed by flow cytometry. Histograms are representative of three independent donors tested. Gray histogram represents cells that were incubated in the absence of cyanine-3-tagged CpG. G, Human neutrophils (2 x 10^6 cells) were preincubated with or without inhibitory CpG-ODN sequences (100 μg/ml). Following preincubation, binding assay was performed with cyanine-3-tagged CpG 2216 (5 μg/ml) for 4 h. Histograms are one representative of three independent donors tested. Gray histogram represents cells that were incubated in the absence of cyanine-3-tagged CpG.
modulating TLR expression, particularly that of TLR9, in human neutrophils.

Because LTB₄ has the capacity to positively modulate TLR9 expression in human neutrophils, we next investigated a possible biological consequence of such an action. We therefore performed binding assays using cyanine-3-tagged CpG-ODN sequences on human neutrophils following a preincubation with LTB₄. As shown in Fig. 1F, a significant binding of tagged CpG was detected using three different concentrations following a 4-h incubation time. Of most interest, tagged-CpG binding was enhanced when neutrophils were preincubated with LTB₄, demonstrating that LTB₄ positively modulates CpG binding on neutrophils. To relate this increase in CpG binding to TLR9 following LTB₄ stimulations, we next performed binding experiments using inhibitory CpG-ODN sequences. As shown in Fig. 1G, CpG binding to human neutrophils was significantly inhibited when cells were pre-treated with inhibitory CpG-ODN sequences, demonstrating a TLR9-dependent binding. The increase in CpG binding mediated by LTB₄ treatment was also inhibited in the presence of inhibitory CpG sequences. In this context lower CpG binding could be detected, providing compelling evidence that LTB₄ modulates TLR9 expression in human neutrophils, resulting in enhanced DNA sequence binding on TLR9. This last set of results provides evidence that LTB₄ contributes to the modulation of TLR9 expression in human neutrophils as an early mechanism for foreign nucleic acid detection.

**LTB₄ potentiates cytokine secretion following CpG-ODN stimulation in human leukocytes**

Because LTB₄ can modulate TLR9 expression on human neutrophils, we investigated whether LTB₄ can potentiate the production of cytokines by human leukocytes in response to a TLR9 ligand. As shown in Fig. 2A, neutrophil stimulation with LTB₄ or CpG-ODN alone did not lead to secretion of TNF-α, as other teams have already reported (32). However, when neutrophils were stimulated with LTB₄ in the presence of CpG-ODN, a marked secretion in TNF-α was observed. A similar secretion pattern was observed for IL-8 (data not shown). Because LTB₄ stimulation leads to potentiation in cytokine secretion by neutrophils following CpG stimulation, we wanted to investigate whether this was also the case in other peripheral blood cellular populations. For that purpose, total PBMC were stimulated with LTB₄ in the presence of absence of CpG. Stimulation of PBMC with LTB₄ also led to an up-regulation in TNF-α secretion in the presence of CpG-ODN (Fig. 2B). As for human neutrophils, PBMC stimulation with a combination of LTB₄ and CpG-ODN led to enhanced IL-8 secretion compared with stimulation with both agonists alone (data not shown). These results demonstrate that LTB₄ potentiates CpG-ODN signaling for enhanced cytokine secretion by neutrophils and PBMC. We next investigated whether potentiation in TNF-α secretion induced by LTB₄ was a specific effect. For that purpose, neutrophils were pretreated or not with the LTB₄ receptor antagonist U75302 (10 μM) before LTB₄ stimulation in the presence of CpG-ODN. As shown in Fig. 2C, U75302 pre-treatment totally abolished TNF-α when both CpG-ODN and LTB₄ stimulations were applied, demonstrating an LTB₄-specific effect. In human neutrophils, potentiation in TNF-α secretion was time dependent, starting as early as 2 h poststimulation and reaching peak secretion at 6 h poststimulation (Fig. 2D). Potentiation in TNF-α secretion by neutrophils stimulated with both LTB₄ and CpG-ODN was also observed as a dose-dependent event reaching statistical significance at a concentration as low as 10⁻⁷ M LTB₄ and reaching peak secretion at 10⁻¹⁰ (10 nM) and 10⁻⁷ M (100 nM) LTB₄ (Fig. 2E). To correlate the enhanced cytokine secretion following stimulation with LTB₄ in the presence of CpG-ODN with enhanced TLR9 expression on the surface of neutrophils, cells were stimulated with CpG-ODN following pretreatment or simultaneous stimulation with LTB₄. Surprisingly, when neutrophils were stimulated simultaneously with
LTB₄ and CpG-ODN, a more elevated secretion of TNF-α was observed as opposed to cells that were pretreated for 30 min with LTB₄ followed by CpG-ODN treatment (Fig. 2F). This result seems to indicate that LTB₄-mediated up-regulation in TLR9 expression levels is unrelated to LTB₄-mediated potentiation in cytokine secretion following CpG-ODN treatment. Taken together, these results demonstrate that LTB₄ stimulation of neutrophils or PBMC potentiates cytokine secretion following stimulation with CpG-ODN, and this potentiation in CpG-ODN stimulation following LTB₄ treatment does not imply an increase in TLR9 expression.

**Potentiation in TNF-α secretion by LTB₄ in human neutrophils and PBMC implicates p38 kinase and JNK**

Our previous results indicate that potentiation in cytokine release following stimulation with LTB₄ and CpG-ODN does not correlate with an up-regulation in TLR9 expression. LTB₄ could therefore activate intracellular events common to its own as well as CpG-ODN signaling pathways. We therefore stimulated neutrophils (Fig. 3A) or PBMC (Fig. 3B) with LTB₄ and CpG-ODN following preincubation with pharmacological inhibitors of intracellular kinases, namely SB203580 against p38 kinase, PD98059 against ERK, SP600125 against JNK, and LY294002 against PI3K. As shown in Fig. 3A, when neutrophils were pretreated with inhibitors directed against p38 and JNK kinases, a dramatic down-modulation in TNF-α secretion was observed as opposed to cells pretreated with pharmacological inhibitors for ERK and PI3K. The dramatic down-modulation in TNF-α secretion when the p38 kinase inhibitor was used was also observed in PBMC (Fig. 3B). To a lesser extent, JNK was also found to participate in the potentiation of cytokine secretion by LTB₄ in CpG-treated PBMC. ERK did not appear to be involved in LTB₄-mediated potentiation in cytokine release in the presence of CpG, because treatment with ERK inhibitor did not abrogate such an effect. Taken together, these data provide evidence that p38 and JNK kinases are two intracellular kinases implicated in LTB₄-mediated potentiation in cytokine secretion in both CpG-stimulated neutrophils and PBMC.

**LTB₄-mediated potentiation in CpG signaling occurs via endosomal-dependent signaling in PBMC, but not in human neutrophils**

In most cases, CpG-mediated signaling is known to occur via TLR9 activation following recruitment of the receptor to endosomal compartments. However, endosomal-independent activation of intracellular signaling events by CpG has also been reported in human leukocytes (19). To verify whether potentiation in CpG-mediated cellular signaling by LTB₄ was related to endosomal signaling, we pretreated human neutrophils (Fig. 4A) or PBMC (Fig. 4B) with an inhibitor of endosomal acidification, chloroquine. As shown in Fig. 4A, chloroquine treatment did not lead to a significant reduction in TNF-α secretion following neutrophil stimulation with LTB₄ and CpG-ODN. In contrast, pretreatment of human PBMC with 5 μM chloroquine resulted in a significant
inhibition in TNF-α secretion following stimulation with LTB₄ and CpG-ODN (Fig. 4B). The use of chloroquine at a higher but nontoxic concentration (20 μM) resulted in almost complete inhibition of TNF-α secretion by PBMC following stimulation with LTB₄ and CpG-ODN. These results demonstrate that potentiation in CpG signaling by LTB₄ requires the implication of endosomes in human PBMC, but not in human neutrophils. To reinforce such an assumption, we performed another set of experiments. TLR9 is known to mobilize in an endosomal compartment and recruit the adaptor molecule MyD88 following ligand binding. MyD88 recruitment to the receptor is the first event involved in intracellular signaling following engagement. Murine splenocytes containing a cell population of mostly lymphoid origin from wild type (WT) as well as mice deficient in MyD88, were stimulated with CpG-ODN in the presence or absence of LTB₄. Spleen cells isolated from mice deficient in another TLR adaptor protein, TRIF, were also used as control. Similar to human PBMC, an up-regulation in TNF-α secretion was present when cells were stimulated with CpG-ODN in the presence of LTB₄ as opposed to stimulation with CpG-ODN alone (Fig. 4C). Although not statistically significant, an up-regulation in TNF-α secretion by murine splenocytes from TRIF−/− mice was also present following stimulation with CpG-ODN in the presence of LTB₄, showing that TRIF is not the predominant TLR adaptor protein molecule necessary for such cell activity. In contrast, in MyD88−/− splenocytes no secretion in TNF-α secretion was detected following stimulation with CpG-ODN in the presence or absence of LTB₄. Together, these results tend to confirm that potentiation in CpG signaling by LTB₄ is an endosomal-independent event in neutrophils as opposed to PBMC, where endosomes as well as MyD88-dependent signaling are necessary for such activity.

Enhanced p38 and JNK activation mediated by LTB₄ following CpG stimulation

Our previous data support the involvement of p38 kinase as well as JNK in LTB₄-mediated potentiation of cytokine secretion following CpG stimulation by both PBMC and human neutrophils. We therefore wanted to investigate whether this potentiation of cytokine secretion was in fact the result of an up-regulation in the activation status of such kinases. For that purpose, neutrophils (Fig. 5A) as well as PBMC (Fig. 5B) were stimulated with LTB₄, CpG-ODN, or a combination of stimuli and the phosphorylation statuses of p38 and JNK were evaluated. Compared with unstimulated cell controls, stimulation of human neutrophils with LTB₄ or CpG-ODN alone lead to a slight but detectable phosphorylation of both p38 kinase and JNK (Fig. 5A), as reported previously (33–35). More interesting was the fact that synergism was observed in p38 and JNK kinase activation when both LTB₄ and CpG-ODN stimulations were performed simultaneously. TAK-1 is an important molecule involved in TLR-dependent signaling. To verify whether the activation of this kinase could also be enhanced in the presence of LTB₄ and CpG-ODN in human neutrophils, other Western blot analyses were performed (Fig. 5A). Although LTB₄ and CpG-ODN alone could lead to a slight phosphorylation of TAK-1, simultaneous stimulation with both ligands led to a stronger activation status for this kinase. These results demonstrate that LTB₄ potentiates TAK-1, p38, and JNK phosphorylation following CpG-ODN stimulation of human neutrophils. In PBMC (Fig. 5B), LTB₄ and CpG-ODN alone lead to a slight up-regulation in TAK-1, p38, and JNK phosphorylation. When cells were stimulated with both ligands, enhanced phosphorylation of the three kinases was also observed, albeit to a lesser extent than in human neutrophils. Taken together, these data provide evidence that LTB₄ can potentiate CpG-ODN signaling in PBMC and neutrophils by leading to increased activation of the TAK-1, p38, and JNK kinases.

Kinase activation by LTB₄ following CpG stimulation does not require endosomal signaling

Our data demonstrated that endosomal signaling was not necessary for potentiation of cytokine secretion by LTB₄ following CpG-ODN stimulation in human neutrophils. However, in PBMC it seems that endosomal signaling is a prerequisite for enhanced cytokine secretion by CpG-ODN stimulation in the presence of LTB₄. We therefore wanted to investigate the necessity of endosomal signaling for kinase phosphorylation in neutrophils and PBMC. To investigate whether this was the case for TAK-1, p38 kinase, and JNK phosphorylation following stimulation of neutrophils or PBMC with CpG-ODN in the presence of LTB₄, cells were pretreated or not with chloroquine before stimulation with different ligands was performed. In human neutrophils, LTB₄ or CpG-ODN alone led to TAK-1, p38 kinase, and JNK phosphorylation either in the presence or absence of chloroquine (Fig. 6A). As suspected, the increase in kinase phosphorylation induced by
the stimulation with both LTB₄ and CpG-ODN was not repressed by chloroquine treatment. Surprisingly, the same pattern was observed in PBMC, where neither LTB₄, CpG-ODN, nor CpG-ODN plus LTB₄ stimulation was affected by chloroquine treatments (Fig. 6B). These results demonstrate that kinase activation by CpG-ODN plus LTB₄ in both neutrophil and PBMC populations does not require endosomal signaling.

Discussion

The development of a successful immune response against invading pathogens relies mostly on the effective detection of such invasion by the host. TLRs are now believed to be crucial sensors for the detection of potentially harmful foreign organisms. By binding to different nucleic acid structures, intracellular TLRs can mount a proper response to aggression. In the present report, we provide evidence that LTB₄ can in fact modulate intracellular TLR9 expression and CpG-mediated signaling for the development of an effective immune response. The underlying mechanism of LTB₄ action on the intracellular TLR system seems to be two edged.

Firstly, we provide evidence that LTB₄ modulates the expression of TLR9 in human neutrophils. TLR9 is known to be necessary for a proper immune defense against bacteria of Streptococcus (36), Klebsiella (37), and Legionella (38) species as well as intracellular pathogens, including viruses such as HSV-2 (39), CMV (40), poxviruses (41), and the parasites Trypanosoma cruzi (8) and Toxoplasma gondii (9). These observations were made using TLR9⁻/⁻ mice. In such mice, infection with all of the aforementioned pathogens was facilitated by the absence of TLR9. LTB₄ has also shown antimicrobial activities against Klebsiella pneumoniae (42), Trypanosoma cruzi (43), and Leishmania amazonensis (44). Moreover, we previously demonstrated the antiviral properties of LTB₄ against different viruses such as human and murine CMV and HSV-1 as well as the influenza virus (20–22). It can therefore be hypothesized that, by modulating TLR9 expression, LTB₄ could lead to enhanced antimicrobial defense against a wide variety of pathogens. In this report, we provide compelling evidence that LTB₄ positively modulates TLR9 expression in human neutrophils and consequently enhances the binding of foreign DNA. Different DNA viruses such as herpesvirus have been known to be recognized by TLR9. In fact, HSV-1 has been shown to possess a considerable amount of CpG sequences (45) and is known to activate TLR9 (26, 46). Knowing the important role played by human neutrophils as front-line cells of the innate immune system, the results reported in the present study suggest a possible new antimicrobial mechanism induced by LTB₄. In fact, by modulating TLR9 expression as well as foreign DNA binding on human neutrophils, LTB₄ could provide a selective advantage to the host for early pathogen detection. Whether this pathogen detection mechanism induced by LTB₄ is a predominant one in vivo, however, needs further investigation.

Secondly, we demonstrated that LTB₄ potentiates CpG-mediated intracellular signaling in PBMC as well as in neutrophils, resulting in enhanced cytokine secretion. CpG oligonucleotides are known to activate TLR9 signaling in different leukocytic cell populations, leading to cytokine secretion (reviewed in Ref. 47). TLR9 signaling is known to be fully dependent on the recruitment of MyD88 to the receptor following ligand binding in endosomal compartments. In PBMC, we demonstrate that LTB₄ in fact leads to enhanced secretion of TNF-α following CpG stimulation in a MyD88- and endosome-dependent manner. In contrast, in human neutrophils it seems that the potentiated secretion of TNF-α when cells were stimulated with a combination of LTB₄ and CpG was completely independent of endosome formation. This observation is in accordance with the growing body of evidence indicating that dsDNA can be recognized in the cytoplasm in a TLR-independent fashion that leads to antiviral cytokine secretion and immune response (48). Evidence that CpG can induce cellular responses independently of endosomal formation has also been gathered in leukocytic cells. In the THP-1 monocytic cell line and monocytederived dendritic cells, a two-step activation model by CpG has been proposed (19). The first step would be the TLR9-independent recognition of CpG at the plasma membrane by an uncharacterized cell surface receptor, leading to the activation of intracellular kinases. These kinases, a second time, would interact with TLR9 and initiate the MyD88-dependent cascade that requires the formation of endosomes. In part, this model resembles the observations we made with PBMC. In PBMC, chloroquine did not abrogate the up-regulation in TAK-1, p38 kinase, and JNK kinase phosphorylation following stimulation with CpG in the presence of LTB₄. However, the up-regulation in cytokine secretion by the stimulation of PBMC with CpG in the presence of LTB₄ was completely dependent on MyD88 as well as endosomal formation. Whether phosphorylation of TAK-1, p38 kinase, and JNK requires...
upstream kinase activation (Src kinase for example) and how endosomal signaling interacts with p38 and JNK for potentiation in cytokine secretion remain to be clarified. The potential signaling events activated following CpG and LTB₄ stimulation in both cell populations are depicted in Fig. 7.

In human neutrophils, a different mechanism seems to be in place due to the fact that both potentiation of cytokine secretion as well as kinase activation by LTB₄ were insensitive to chloroquine treatments. It is already known that bacterial DNA can be recognized by neutrophils via a CpG- and TLR9-independent mechanism, leading to cell activation (49). Other evidence has also been documented concerning TLR9-independent activation of B cells and dendritic cells by DNA (18, 50). Recently, a potential cytoplasmic DNA receptor named DAI (DNA-dependent activator of IFN regulatory factors, also named DLM1/ZBP1) has been identified (51). Whether the potentiation in cytokine secretion induced by LTB₄, following neutrophil CpG stimulation implicates DAI or other potential intracellular DNA sensors remains, however, to be determined.

In this report, we provide evidence that signaling induced by LTB₄ can intersect CpG signaling at the level of TAK-1 activation, leading to enhanced cytokine secretion. TAK-1 expression is already known to be central to most, if not all, signaling induced following TLR activation, whether it be TLRs using MyD88 or TLRs using TRIF as their adaptor protein (52). Studies using TAK-1-deficient cells have shown that this protein is essential for TLR response, CD40, and B cell receptor crosslinking (53). The immune response induced by Ag stimulation is also impaired in TAK-1-deficient cells. TAK-1 is also important in the regulation of the development, survival, and function of T cells (54) as well as thymocyte development (55). These examples show how important TAK-1 is to innate and adaptive immunity. TAK-1 kinase activity is also known to be central to antimicrobial immunity against Yersinia enterocolitica (56). With all of the aforementioned activities related to TAK1, it is therefore not surprising to observe that LTB₄ in fact modulates TAK-1 activation by itself as a potential mechanism for an enhanced immune defense. Moreover, LTB₄ potentiates TAK-1 activation following CpG treatment in both neutrophils and PBMC. It is therefore possible to hypothesize that LTB₄ could have the potential to modulate the signaling of some or all TLR members at the level of TAK-1 activation. This activity might be specific to the concentration of the TLR ligand used, the cell type studied, as well as the immune response tested. However, more investigations are needed to clarify this point.

In this report we demonstrated that LTB₄ can potentiate CpG signaling in human neutrophils as well as PBMC for enhanced cytokine secretion. Modulation of TLR signaling can now be added to the immunomodulatory properties associated to LTB₄ and can be seen as another strategy to boost the immune system.

Acknowledgments
We thank Pierrette Côté for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.

References
2658 LTB4 ENHANCES CELL RESPONSE TO TLR9 LIGAND
like receptor expression reveals CpG DNA as a unique microbial stimulus for
plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high
30. Theron, A. J., C. M. Gravett, H. C. Steel, G. R. Tintinger, C. Feldman, and
activates dendritic cells via TLR9-dependent and -independent pathways.
77: 71–79.
61–68.
172: 4926–4933.
receptors stimulate
plasmacytoid dendritic cells and
and murine dendritic cells through a Toll-like receptor 9-dependent pathway.
receptors stimulate
plasmacytoid dendritic cells and
and murine dendritic cells through a Toll-like receptor 9-dependent pathway.
receptors stimulate
plasmacytoid dendritic cells and
and murine dendritic cells through a Toll-like receptor 9-dependent pathway.
receptors stimulate
plasmacytoid dendritic cells and
and murine dendritic cells through a Toll-like receptor 9-dependent pathway.
receptors stimulate
plasmacytoid dendritic cells and
and murine dendritic cells through a Toll-like receptor 9-dependent pathway.