Leukotriene B_4 Triggers the In Vitro and In Vivo Release of Potent Antimicrobial Agents[1]

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Leukotriene B_4 (LTB_4) is a bioactive lipid derived from the metabolism of arachidonic acid. Mainly produced by polymorphonuclear leukocytes (PMN) and macrophages, LTB_4 triggers several functional responses important in host defense, including the secretion of lysosomal enzymes, the activation of NADPH oxidase activity, NO formation, and phagocytosis. We report that LTB_4, but not structural analogs thereof, stimulates primed human PMN to release molecules having potent antimicrobial activities. Exposure of bacteria (*Escherichia coli* and *Staphylococcus aureus*) or viruses (herpes simplex virus type 1 and HIV type 1) to supernatants of LTB_4-activated PMN lead to ≥90% reduction in infectivity. ELISA and mass spectroscopy analysis of proteins released from LTB_4-activated PMN have identified several antimicrobial proteins, including α-defensins, cathepsin G, elastase, lysozyme C, and LL-37, that are likely to participate in the killing of microorganisms. In addition to these in vitro observations, i.v. injections of LTB_4 (50 μg/kg) to monkeys led to an increase in α-defensin plasmatic levels and enhanced ex vivo antimicrobial activities of plasma. These results demonstrate the ability of LTB_4 to cause the release of potent antimicrobial agents from PMN in vitro as well as in vivo and add further support to the important role of LTB_4 in host defense. ***The Journal of Immunology,*** **2007, 178: 8036–8045.**

The human body constantly fights invading pathogens through a plethora of immune defense mechanisms. Among the effector cells playing a role in both antibacterial and antiviral defense are the polymorphonuclear leukocytes (PMN).[3] The PMN is the most abundant circulating leukocyte and the first cell type to emigrate from blood vessels and reach infectious foci in the periphery. Neutrophil's functions include the phagocytosis of particles, the production of reactive oxygen species (O_2^- and H_2O_2), and the production of lipid mediators (leukotrienes, lipoxins, prostaglandins, and platelet activating factor), lactic acid, and IFNs as well as the secretion of antimicrobial proteins such as lysozyme, defensins and, cathelicidins (1, 2), which directly destroy microorganisms. Defensins are small cationic peptides (30 aa in mature form) with three disulfide bridges (3–5). They are the most abundant proteins within cytoplasmic azurophilic granules of PMN. Two main defensin subfamilies, α and β, exist in humans. α-Defensins are found in PMN, Paneth cells, and a few types of epithelial cells, whereas β-defensins are present in epithelial cells. Defensins have antimicrobial activities against fungi, bacteria, and viruses as well as abilities to neutralize lethal anthrax toxin (6–12). α-Defensins also facilitate and amplify the innate and adaptive immune responses because they can attract human $CD4^+CD45RA^+$ or $CD8^+$ T cells (13, 14), immature dendritic cells (14), and monocytes (15). α-Defensins induce the release of IFN-γ, IL-6, and IL-10 from T cells, TNF-α, and IL-1β from monocytes, and IL-8 from alveolar macrophages and intestinal and lung epithelial cell-lines (16–21). α-Defensins are chemotactic for monocytes, naive $CD4^+$ T cells, $CD8^+$ T cells, and PMN and exert their effects through an unknown receptor.

Activated PMN not only secrete antimicrobial peptides/enzymes and chemokines/cytokines but also bioactive lipids. One important and well characterized lipid is leukotriene (LT) B_4, a tetraunsaturated 20-carbon chain fatty acid derived from the oxidative metabolism of arachidonic acid through the successive actions of the 5-lipoxygenase and the leukotriene A_4-hydrolase enzymes (for review see Ref. 22). LTB_4 activates, through autocrine and paracrine mechanisms, several leukocyte types by binding and signaling through at least two distinct cell surface G protein-coupled receptors, BLT1 and BLT2 (23–25). The LTB_4 receptors are expressed in monocytes, granulocytes, lymphocytes, and in several hemopoietic cell lines (23–25). LTB_4 stimulation leads to a number of functional responses important in host defense such as the secretion of lysosomal enzymes, the activation of NADPH oxidase activity, NO formation, and phagocytosis. LTB_4 also stimulates the expression of the β2-integrin (CD11b/CD18), an effect likely related to its ability to stimulate leukocyte migration and phagocytosis. Lastly, LTB_4 is reported to increase NK cells cytotoxicity (26, 27) and to activate B lymphocyte proliferation and Ab formation in vitro (28).

In a previous study we made the observation that LTB_4 triggers the in vitro release of α-defensins from human PMN, including those isolated from HIV-1-infected individuals; in the same study we also reported that the i.v. injection of LTB_4 in man causes a marked increase in the plasma α-defensin level (29). In the present work, we extend these studies and provide evidence that supernatants from LTB_4-activated human PMN contain potent antibacterial and antiviral substances. Furthermore, we show that plasma of

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; LB, Luria-Bertani; LT, leukotriene.

monkeys injected (i.v. bolus) with LTB_4 express enhanced ex vivo antimicrobial activities relative to control plasma samples, highlighting the important contributions and potential usefulness of LTB_4 in the fight against pathogens.

Materials and Methods

Cell lines, viruses, and bacteria

Vero cells were obtained from the American Type Culture Collection and cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), L-glutamine, and penicillin-streptomycin. TZM-bl (30), a modified HeLa cell derivative susceptible to infection by M- (R5) and T-tropic (X4) isolates of HIV-1, was obtained form the National Institutes of Health AIDS Repository Reagent Program (Germantown, MD). Cells were passaged by trypsinization of the cellular monolayer. HSV-1 (MacIntyre strain) was propagated by infecting Vero cells at a multiplicity of infection of 0.01. When cellular destruction was near maximal (3–4 days), the supernatant was collected, filtered (0.45 μm), and centrifuged at 30,000 $\times$ *g* for 90 min to pellet the viruses. The viral pellet was resuspended in DMEM medium without serum. HSV-1 titer was determined by a standard plaque assay on Vero cells. The infectious HSV-1 titer was estimated at 5×10^7 PFU/ml. HIV-1 particles were produced by transient transfection in human embryonic kidney 293T cells, as previously described (31). Infectious molecular clones of HIV-1 that were used in this study include pNL4-3 (a prototypic X4-tropic variant) and pNL4-3bal*env* (R5-tropic). The pNL4-3bal*env* vector was generated by replacing the *env* gene of the T-tropic HIV-1 strain NL4-3 with that of the macrophage-tropic HIV-1 Bal strain, thus resulting in an infectious molecular clone with macrophage-tropic properties (kindly provided by R. Pomerantz, Thomas Jefferson University, Philadelphia, PA) (32). The NL4-3 molecular construct was obtained through the National Institutes of Health AIDS Repository Reagent Program. The virus-containing supernatants were filtered through a 0.22-μm cellulose acetate syringe filter and normalized for virion content using an in-house, sensitive, double-Ab sandwich ELISA specific for the viral $p24^{gag}$ protein (33). All virus preparations underwent only one freeze-thaw cycle before their use in subsequent experiments.

Single colonies of both *Escherichia coli* and *Staphylococcus aureus* were grown overnight in Luria-Bertani (LB) broth. The overnight cultures were diluted 1/100 in fresh LB broth and the bacteria were grown to an OD (600 nm) of 0.5. The cells were pelleted, washed twice with a phosphate buffer (0.1 M at pH 7.4) and resuspended in buffer at a concentration of 500 CFU/ml.

Isolation of human PMN

Venous blood from healthy volunteers was collected in tubes containing heparin and PMN were isolated as previously described (34). The PMN suspension contained mainly neutrophils (95%) with eosinophils as the major contaminant, and cell viability was always >98% as measured by trypan blue exclusion. PMN were resuspended at 1×10^6 cells/ml in M199 medium without serum or antibiotics unless stated otherwise.

Reagents

LTB_4 and the enantiomer of LTB4 were obtained from Cascade Biochem. LTE_4, 12-epi-LTB_4, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4, 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), 20,20,20-trifluoro-LTB_4, 20-OH-LTB_4, 5-hydroxyeicosatetraenoic acid (5-HETE), and LTB_4 ELISA kits were purchased from Cayman Chemical. LTB_4 and analogs stock solutions were in ethanol and used at 1 nM to 1 μM to stimulate PMN. Cytochalasin B was purchased from Sigma-Aldrich and dissolved in DMSO. HNP-1 ELISA kits were obtained from Hycult Biotechnology. Untreated (control) PMN were incubated with corresponding volumes of ethanol and DMSO.

Stimulation of human PMN and antimicrobial and α-defensin assays

Freshly isolated human PMN were preincubated (10×10^6/ml) in M199 medium containing 10 μM cytochalasin B for 15 min at 37°C, after which LTB_4 or analogs were added to each tube to various concentrations (0, 10^{-8}, 10^{-7}, and 10^{-6} M). Incubations were stopped at different times (as indicated in figure legends) by transferring tubes in an ice/water bath. Cell suspensions were centrifuged (250 $\times$ *g* for 10 min at 4°C) and cell-free supernatants were collected and assayed for virucidal activity as follows. One or 10 μl of PMN supernatants were diluted with RPMI 1640 medium to a final volume of 100 μl. Varying quantities of infectious HSV-1 (500–15,000 PFU/50 μl) were added to the diluted PMN supernatants and the mixtures were incubated at 37°C for periods of time varying between 1 and 30 min; the volumes were adjusted to 0.5 ml with M199 medium and the mixtures were added to Vero cell monolayers to determine HSV-1 infectivity by a standard plaque assay. α-Defensins were directly assayed in the appropriately diluted cell-free supernatants by ELISA according the manufacturer's instructions.

To test for anti-HIV-1 activity, 0.15 ml of supernatants from control and LTB_4-activated PMN were mixed with 0.05 ml of T- or M-tropic HIV-1 viral stock (10 ng p24 equivalent) and incubated for 1 h at 37°C. Viral infectivity was then determined by adding the solutions to the wells of a 96-well plate coated with TZM-bl indicator cells (30). Virions were allowed to infect cells for 1 h after which the wells were washed twice with PBS followed by the addition of 0.2 ml of culture medium. Infection was allowed to proceed for 48 h after which the cells were lysed and luciferase activity was determined.

To test for bactericidal activity, PMN were primed with 10 μM cytochalasin B for 30 min followed by LTB_4 stimulation for 1 h. One-half milliliter of cell-free supernatants was incubated for 2 h at 37°C with 0.5 ml of exponentially grown *E. coli* or *S. aureus* resuspended in potassium phosphate buffer (0.1 M at pH 7.4). The bacteria were then spun down, resuspended in 0.1 ml LB broth, plated on LB-agar plates, and incubated at 37°C for 18 h after which the numbers of colonies were determined.

Monkey studies

Four young adult ovariectomized female cynomolgus monkeys (*Macaca fascicularis*) weighing between 2.8 and 4.5 kg were used for the study. A single 2-ml bolus of LTB_4 (50 μg/kg) was administered to anesthetized (10 mg/kg ketamine and 10 μg/kg glycopyrrolate i.m.) monkeys by i.v. injection. LTB_4 was administered as an aqueous isotonic and sterile solution of the sodium salt containing a 25 mM phosphate buffer and sodium chloride. Blood samples (~1.5 ml/time point) were collected at various time points over a period of several hours before and after LTB_4 administration (−5 to +840 min). The blood samples were collected in tubes containing EDTA as the anticoagulant, immediately mixed, and kept in an ice water bath for a maximum of 20 min. The samples were centrifuged (4°C) and the plasma was collected and stored at −80°C until it was analyzed for LTB_4 and α-defensin contents by ELISA. The same blood samples were used to monitor absolute blood neutrophil counts by flow cytometry. Plasmatic LTB_4 and α-defensin analyses by ELISA were performed directly on appropriately diluted plasma samples without prior treatment. As a consequence, it is likely that the measured levels of LTB_4 are overestimated, especially the basal levels. For these reasons, plasmatic LTB_4 levels are expressed as arbitrary units.

Ex vivo antimicrobial activity of monkey plasma samples

Freshly isolated plasma samples (t = −5, +120, and +240 min) from monkeys that had received an i.v. bolus of LTB_4 were tested for anti-HSV-1 and antibacterial activities. For anti-HSV-1 activity, 10-μl aliquots of plasma (in triplicate) were incubated with 10 μl of HSV-1 (1,500 PFU) for 1 h at 37°C. The mixtures were then diluted to 0.5 ml with M199 medium and added to the wells of a 12-well plate containing Vero cells plated 1 day earlier. Viral infectivity was determined by a standard plaque assay. For antibacterial testing, monkey plasma aliquots (20 μl) were mixed in sterile Eppendorf tubes with 20 μl (~500 CFU) of exponentially grown and PBS-washed *E. coli* or *S. aureus* for 2 h at 37°C under constant agitation (100 rpm). After the incubation period, 60 μl of LB broth was added to each tube and the mixtures were spread onto LB-agar plates. After an overnight incubation at 37°C, the number of colonies on each plate was determined.

LC-MS/MS identification of selected proteins released from LTB4-activated PMN

Purified human PMN (50×10^7/sample) were incubated in serum-free medium supplemented with cytochalasin B (10 μM) for 10 min. Cells were then treated with 100 nM LTB_4 (or ethanol) for 5 min after which the cell-free supernatants were collected and processed as described (35). The proteins were separated by denaturing gel electrophoresis and visualized by Coomassie blue staining. The major proteins (the one we could visualize) that had been released upon PMN stimulation by LTB_4 were cut out of the gels. Isolated proteins were digested with trypsin and the tryptic peptides were resolved by reversed phase HPLC and analyzed by mass spectrometry (LCQ Deca XP liquid chromatography/tandem mass spectrometry; Thermo Scientific) at the Centre Protéomique de l'est du Québec (Centre Hospitalier de l'Université Laval Research Center, Quebec, Canada).

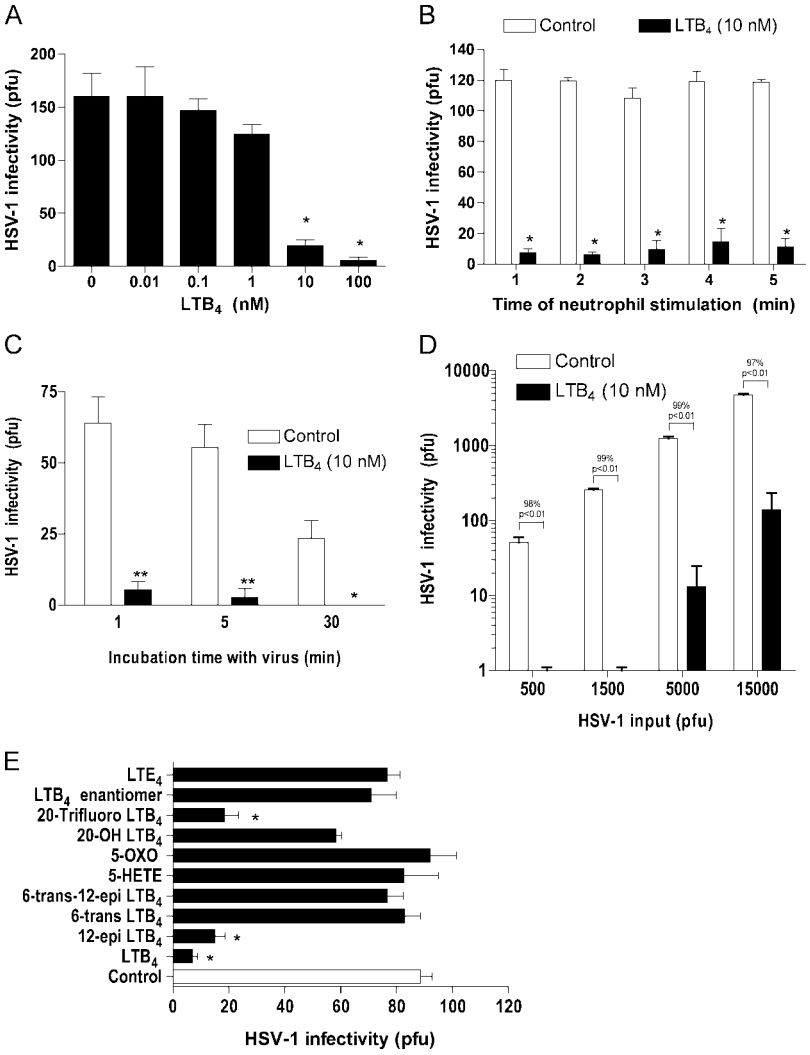

FIGURE 1. LTB$_4$ causes the release of anti-HSV-1 molecules from PMN. *A*, Human PMN were stimulated for 1 min with increasing concentrations of LTB4 and the cell-free supernatants were obtained. Ten microliters of supernatants were added to 100 PFU of infectious HSV-1 (150-μl final volume). HSV-1 suspensions were then incubated for 1 h at 37°C, followed by viral infectivity determination using a standard plaque assay. *B*, Human PMN were stimulated with 10 nM of LTB4 for periods of time varying between 1 and 5 min, after which the cell-free supernatants were collected. Ten microliters of supernatants were added to 100 PFU of infectious HSV-1 (150-μl final volume) for 1 h at 37°C, followed by viral infectivity determination using a standard plaque assay. *C*, Ten microliters of unstimulated or LTB$_4$-activated (10 nM) PMN supernatants were added to 100 PFU (150-μl final volume) of HSV-1 suspensions and incubated for periods of time varying from 1 to 30 min. Viral infectivity was then determined by plaque assay. *D*, Ten microliters of unstimulated or LTB$_4$-activated (10 nM) PMN supernatants were added to increasing quantities of infectious HSV-1 (500–15,000 PFU/150-μl final volume). Viral suspensions were incubated for 1 h at 37°C, after which viral infectivity was determined by plaque assay. *E*, PMN were stimulated with 100 nM LTB$_4$ or structurally related compounds for 5 min, after which the supernatants were collected and assayed for antiviral activity. Results presented are expressed as mean ± SD from triplicates and are representative of at least three independent experiments (*, $p < 0.05$; **, $p < 0.01$).

Statistical analysis

Statistical analysis was performed with the aid of the GraphPad InStat 3 software using Student *t* test with Welch correction; data were considered significant when the $p < 0.05$.

Results

Supernatants from LTB$_4$-activated PMN efficiently neutralize viruses and bacteria

PMN are among the first cells to emigrate from blood vessels toward infected foci. Upon pathogen encounter, PMN secrete antimicrobial agents and perform phagocytosis. In the present work we sought to define the ability of LTB$_4$ to induce the release of antimicrobial agents in vitro and in vivo. Our first series of experiments were designed to address whether LTB$_4$ can cause PMN to produce and/or secrete substances that could neutralize infectious agents. To do so, PMN were stimulated with increasing concentration of LTB$_4$ (0.01–100 nM) for 1 min, after which the cell-free supernatants were collected and assayed for anti-HSV-1 activity. The results obtained (Fig. 1*A*) indicate that 1–10 nM of LTB$_4$ were required to trigger the release anti-HSV-1 molecules. Concentrations of LTB$_4$ higher than 10 nM caused maximal release of anti-HSV-1 molecules, whereas stimulation of PMN with ≤1 nM LTB$_4$ had marginal effects. To determine the kinetics of the release of anti-HSV-1 by PMN following LTB$_4$ stimulation, we performed a time course study. PMN were stimulated with LTB$_4$ for time periods ranging between 1 and 5 min, after which the cell-free supernatants were assayed for anti-HSV-1 activity (Fig. 1*B*). Maximal release of antiviral substances was reached after a 1-min stimulation with LTB$_4$, with longer stimulation periods (up to 5 min) causing no further increase in release of antiviral substances. We next sought to determine the incubation time needed for HSV-1 particle inactivation by the supernatants from LTB$_4$-stimulated PMN (Fig. 1*C*). The results obtained indicate that a 1-min incubation of the virus (500 PFU/135 μl) with 10 μl of supernatant (1/15 final dilution) from LTB$_4$-activated PMN is sufficient to reduce the number of infectious viral particles by 90% ($p < 0.001$) when compared with that of mock supernatants (from control PMN). Longer incubation times further destroyed HSV-1 infectivity with complete viral inactivation observed after 30 min of incubation. It is also important to note that control PMN spontaneously secrete, albeit at a much lower concentration compared with LTB$_4$-activated PMN, anti-HSV-1 molecules that, over time, reduce viral infectivity. The amount of spontaneous and LTB$_4$-triggerred antiviral substances released by PMN varied ~2–3-fold between blood donors.

We next determined how many HSV-1 particles could be inactivated by the supernatants from LTB$_4$-activated PMN (Fig. 1*D*). The results indicate that a >95% reduction in HSV-1 infectivity titer was recorded when the viruses (500–15 000 PFU) are incubated in the presence of 10 μl of LTB4-stimulated PMN. When a 1-μl aliquot of supernatant from LTB$_4$-activated PMN was tested,

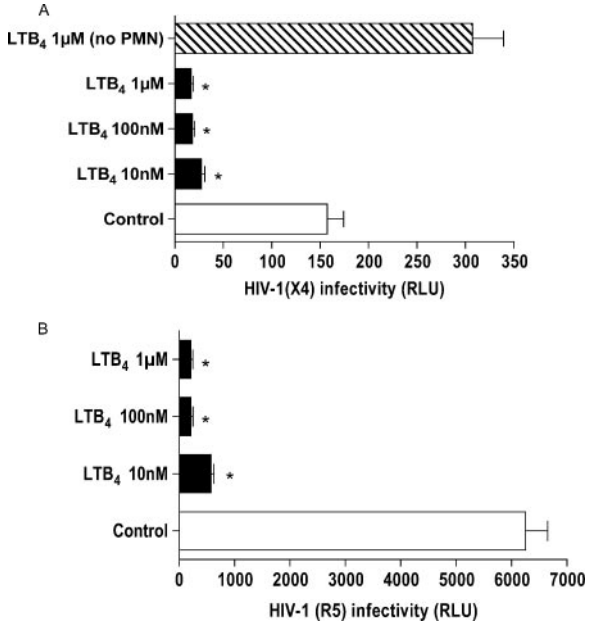

FIGURE 2. LTB_4 causes the release of anti HIV-1 molecules from PMN. Human PMN were stimulated for 5 min with increasing concentrations of LTB_4 or its diluent (control), after which cell-free supernatants were obtained. One hundred fifty microliters of cell-free supernatants were added to 10 ng of p24 equivalent in 50 μl of T(X4)-tropic 9 (*A*) or M(R5)-tropic (*B*) isolates of HIV-1 in a final volume of 200 μl, and the viral suspensions were incubated for 1 h at 37°C. Viral infectivity was then determined by infection of the TZM-bl reporter cell line. Forty-eight hours later, cells were lysed and the luciferase activity determined. Results are expressed as mean ± SD relative luciferase units (RLU) from triplicates and are representative of two experiments ($p < 0.01$).

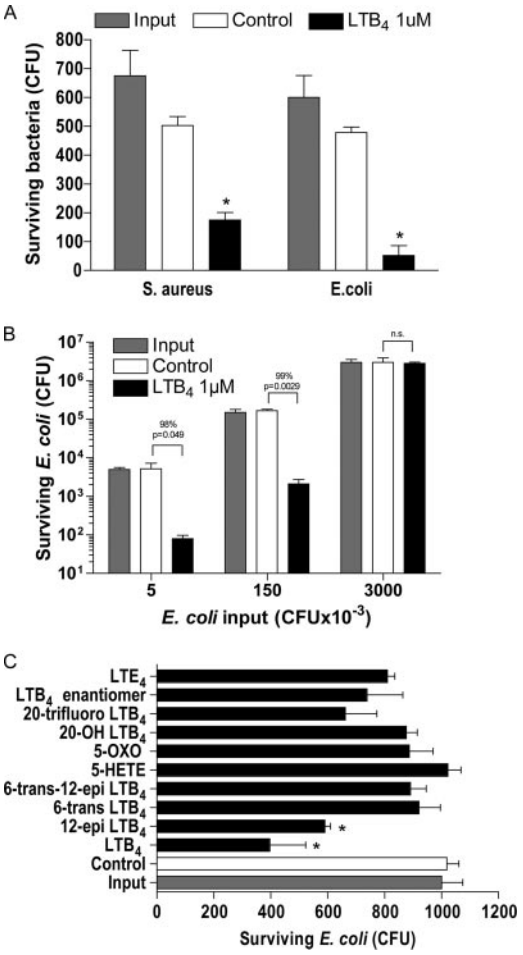

FIGURE 3. LTB_4 causes the release of antibacterial molecules from PMN. Human PMN were stimulated for 5 min with 1 μM LTB_4 or the diluent (control) and cell-free supernatants were obtained. *A*, One half-milliliter aliquots of cell-free supernatants were added to ~500 exponentially growing *E. coli* or *S. aureus* bacteroa in a final volume of 1 ml. After an incubation of 1 h at 37°C, bacteria were spread on LB agar plates and colonies were counted after overnight growth at 37°C. *B*, One half-milliliter aliquots of cell-free supernatants were added to increasing numbers of exponentially growing *E. coli* in a final volume of 1 ml. After an incubation of 1 h at 37°C the number of viable bacteria was determined as described above. *C*, PMN were stimulated with 100 nM LTB_4 or analogs for 5 min, after which the supernatants were assayed for anti-*E. coli* activity (input of 1000 CFU) as described above. Results presented are expressed as mean ± SD from triplicates and are representative of at least two independent experiments (*, $p < 0.05$).

mean reductions in viral titers of 65, 46, and 23% were recorded for viral inputs of 500, 1,500 and 5,000 PFU, respectively (data not shown).

To assess the specificity of the stimulatory effect of LTB_4 on the release of antimicrobial molecules, PMN were incubated as described above with structural analogs of LTB_4. The cell-free supernatants were subsequently tested for anti-HSV-1 activity. The results presented in Fig. 1*E* indicate that of the various compounds tested, LTB_4 is the most potent inducer of antimicrobial activity from PMN, followed closely by the 12-epi-LTB_4 analog. It is important to note here that the 12-epi-LTB_4 used in this experiment (as well as in other experiments reported herein) has been found to contain 2.6% LTB_4 by reverse-phase HPLC analysis (data not shown), which may account, at least in part, for the observed activity of this compound. The 20, 20, 20-trifluoro-LTB_4 analog also triggered the release of anti-HSV-1 compounds while all other analogs tested were unable to induce the release of virucidal molecules, when assayed at 100 nM. These data are in agreement with previous studies where 12-epi-LTB_4 and 20,20,20-trifluoro-LTB_4 were reported to show significant biological activities, although lower than those of LTB_4 (36, 37), while several other analogs were found to ≥100-fold less active than LTB_4 (38).

The antimicrobial activity of supernatants from LTB_4-activated PMN was also tested on M- and T-tropic isolates of HIV-1. The results (Fig. 2) indicate that the infectivity of both the T- (Fig. 2*A*) and the M-tropic variant (Fig. 2*B*) can be efficiently neutralized by compounds released from LTB_4-activated PMN. Incubation medium containing 1 μM LTB_4 but not exposed to PMN had no effect on HIV-1 infectivity, therefore indicating that PMN are essential for the observed effects.

We next determined whether the supernatants from LTB_4-activated PMN could also neutralize bacteria. The results obtained (Fig. 3*A*) indicate that the supernatants form LTB_4-stimulated PMN do display bactericidal activities against *E. coli* and *S. aureus*, with the former being killed more efficiently than the latter. We next assessed the antibacterial potency of PMN supernatants by incubating a fixed volume (0.5 ml) of untreated or LTB_4-activated (1 μM) PMN supernatants with increasing numbers of *E. coli*. The results (Fig. 3*B*) obtained indicate that 0.5 ml of supernatants from LTB_4-activated PMN can effectively (99%) kill up to $1{,}5 \times 10^5$ *E. coli* bacteria. No bactericidal activity was noted when supernatants were incubated with a higher concentration of bacteria (i.e., 3×10^6).

To determine whether the release of antimicrobial molecules is specific to LTB_4, PMN were stimulated with eicosanoids structurally related to LTB_4 and the cell-free supernatants were tested for

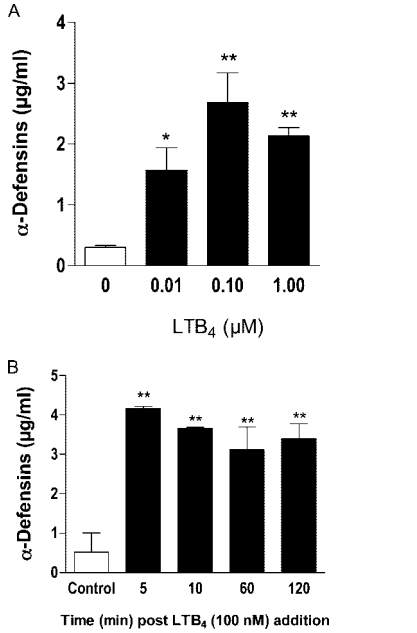

FIGURE 4. LTB$_4$ triggers the release of α-defensins from human PMN. *A*, Human PMN were stimulated for 30 min with LTB$_4$ (0.01–1 μM) or its diluent and cell-free supernatants were assayed for α-defensin content using a commercial ELISA. *B*, Human PMN were stimulated for varying periods of time (1–120 min) with 100 nM LTB$_4$ or its diluent (control) and cell-free supernatants were assayed for α-defensin content. Results are expressed as mean ± SD from triplicate incubations from one experiment representative of at least three experiments with different blood donors (*, $p < 0.05$; **, $p < 0.01$).

antimicrobial activity. The results presented in Fig. 3*C* indicate that, of the various compounds tested, LTB$_4$ is the most potent inducer of antimicrobial activity from PMN. The 12-epi-LTB$_4$ analog also triggered the release of antimicrobial compounds, but less efficiently than LTB$_4$. Other analogs were unable to induce the release of bactericidal molecules at the 100 nM concentration tested.

In vitro release of α-defensins following PMN stimulation with LTB$_4$

The results obtained so far clearly demonstrate that LTB$_4$ effectively triggers the release of antimicrobial compounds form human PMN. Considering that the release of antimicrobial agents by LTB$_4$ occurs within 1 min and is thus compatible with a release of

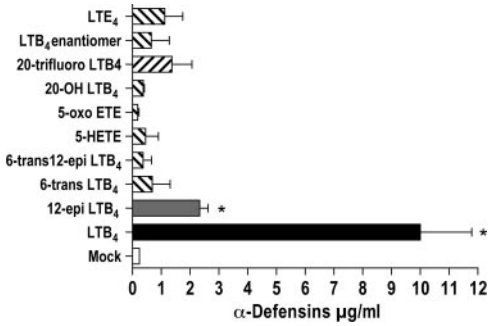

FIGURE 5. Specificity of the effect of LTB$_4$ on α-defensin release by PMN. Human PMN were incubated with LTB$_4$ and 10 structurally related eicosanoids, all at 100 nM for 5 min, and cell-free supernatants were assayed for α-defensin content using a commercial ELISA. Results are expressed as mean ± SD from triplicate incubations from one experiment representative of two independent experiments (*, $p < 0.05$).

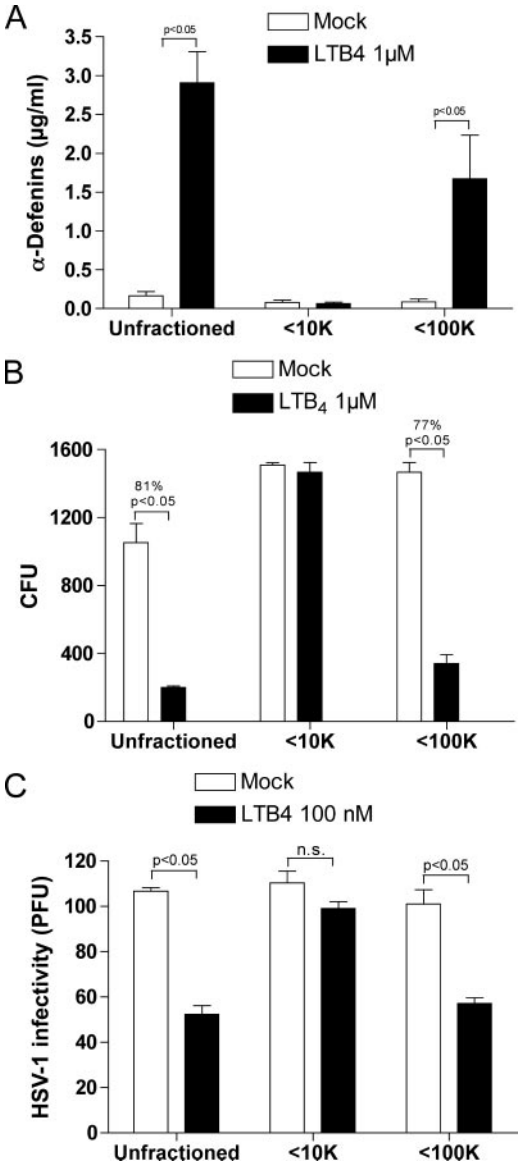

FIGURE 6. Fractionation of antimicrobial activity released from LTB$_4$-activated PMN. Human PMN were stimulated with LTB$_4$ (1 μM) or its diluent (control) for 1 h and supernatants were either used directly or fractionated by centrifugation on YM-10 or YM-100 Microcon filter units. Filtrates were assayed for α-defensin content (*A*), antibacterial (*E. coli*) activity (*B*), or anti-HSV-1 activity (*C*). Results are expressed as mean ± SD of triplicate assays from one experiment representative of two independent experiments.

stored agents, we next investigated whether LTB$_4$ induction could trigger PMN degranulation and α-defensin release. PMN (99% pure) were incubated with increasing concentrations of LTB$_4$ for 30 min and cell-free supernatants were assayed for α-defensin content. The results obtained (Fig. 4*A*) indicate that 10 nM LTB$_4$ induces a significant release of α-defensins from PMN. Maximal levels (9-fold increase over untreated cells) of α-defensins were observed in response to 100 nM LTB$_4$. We next studied the kinetics of α-defensin release from LTB$_4$-stimulated PMN. Results (Fig. 4*B*) indicate that α-defensin release is already maximal 5 min after LTB$_4$ stimulation, in agreement with the fact that α-defensins are preformed and stored in cytoplasmic granules that are rapidly secreted upon PMN activation.

To determine the specificity of the effect of LTB$_4$ on α-defensin release, PMN were incubated for 30 min with structurally related

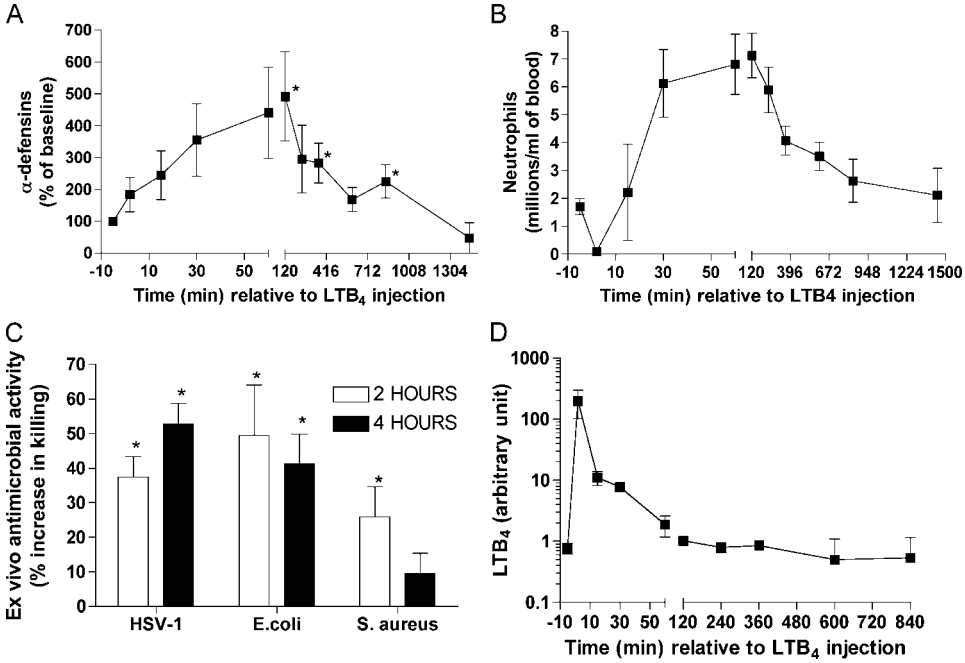

FIGURE 7. Effects of i.v. LTB_4 injection in monkeys on plasmatic α-defensin levels and antimicrobial activities. Monkeys ($n = 4$) were injected i.v. with LTB_4 (bolus 50 μg/kg) and blood samples were obtained at various time points using EDTA as the anticoagulant. *A*, Plasmatic α-defensin levels were determined using a commercial human α-defensin ELISA kit that cross-reacts with macaque α-defensins. The standard curve was generated using human α-defensins 1–3. α-Defensin levels are expressed as mean change relative to baseline levels (mean 11 ng/ml) ± SEM. (∗, $p < 0.05$). *B*, Circulating neutrophil counts at various time points relative to LTB_4 injection were measured by flow cytometry. Results are expressed as mean ± SEM. *C*, Plasma samples were obtained before ($t = -5$ min) and 2 and 4 h after LTB_4 injections and were immediately tested for anti HSV-1, anti-*E. coli*, or anti-*S. aureus* activities. Results are expressed as mean increase ± SD of antimicrobial activity relative to the control plasma samples ($t = -5$ min). Antimicrobial activity of control plasma samples varied between 10 and 20%. The antimicrobial activity of each plasma sample was measured in triplicate as described under *Materials and Methods* (∗, $p < 0.05$). *D*, Plasmatic LTB_4 levels relative to time of injection. Plasma samples were obtained from monkeys before ($t = -5$ min) or at several time points relative to LTB_4 i.v. injection. Plasmatic LTB_4 levels were determined by ELISA as described under *Materials and Methods* and the results are expressed as mean ± SD LTB_4 levels (arbitrary units).

eicosanoids (100 nM), after which the cell-free supernatants were assayed for α-defensin content. A weak induction of α-defensins was seen upon treatment with 12-epi-LTB_4 only, and none of the other analogs tested triggered a release of α-defensins comparable to the one seen with LTB_4 (Fig. 5).

Antimicrobial peptides are responsible for the antimicrobial activity found in LTB_4-activated PMN supernatants

Considering that PMN generate low molecular mass microbicidal agents (such as hydrogen peroxide and NO), we attempted to determine the relative importance of these agents vs peptidic microbicides in the antimicrobial activities of LTB_4-treated PMN supernatants. We used Microcon (Millipore) centrifugation devices (microporous cellulose filters) to fractionate PMN supernatant components on the basis of their molecular mass. When PMN supernatants were centrifuged over YM-10 Microcon filter units, which theoretically allow molecules of <10 kDa to pass through the filter, most of α-defensins were eliminated from the filtrate (Fig. 6*A*). This suggests that α-defensins, which have a molecular mass of ~3 kDa, might have aggregated or been retained by the filter as a consequence of their carbohydrate-binding properties. In fact, when recombinant α-defensins 1–3 were centrifuged through the YM-10 Microcon filter units, >70% of α-defensin input was retained by the filter (data not shown). In contrast, the centrifugation of supernatants over YM-100 columns, which allow molecules of <100 kDa to pass through the filter, had minimal effects on α-defensin concentration in the filtrate. These filtrates were then tested for antimicrobial activities against *E. coli* (Fig. 6*B*) and HSV-1 (Fig. 6*C*). The results obtained indicate that unfractionated supernatants have both antibacterial and antiviral activities. However, filtrates of supernatants on YM-10 filters completely lost their antibacterial and antiviral activities. Conversely, filtrates of supernatants spun through YM-100 filters retained antibacterial and antiviral activities equivalent to unfractionated supernatants. These results suggest that antimicrobial proteins such as α-defensins, but not small molecular mass microbicides, constitute most of the antibacterial and antiviral activities observed in LTB_4-treated PMN supernatants.

We next analyzed untreated and LTB_4-activated PMN supernatants by gel electrophoresis, and proteins were visualized by Coomassie blue staining (data not shown). LTB_4 stimulation enhanced the secretion of several proteins of molecular masses ranging from 5 to 150 kDa. These bands were excised from the gel and identified by mass spectrometry. Several proteins were readily identified, many of which are reported as having antimicrobial activities. These include the serine proteases cathepsin G, elastase, and proteinase 3, the muramidase lysozyme C, and the antimicrobial peptides CAP-18/LL-37. Other identified proteins include lactoferrin and a matrix metalloprotease (MMP-9). α-Defensins were secreted in quantities that were insufficient to be visualized by Coomassie blue staining but were easily measurable by ELISA.

Increase in α-defensin plasmatic levels following i.v. injection of LTB_4 to monkeys

We next investigated whether LTB_4 could trigger the release of antimicrobial proteins in animals. LTB_4 was injected into four macaques (i.v. bolus at 50 μg/kg) and blood samples were collected

to allow measurements of α-defensin levels in plasma and the determination of the number of circulating PMN. The results obtained (Fig. 7) indicate that LTB_4 injections do indeed cause an elevation of the plasmatic α-defensin level (as measured by ELISA). Although there were differences between animals in the magnitude of the response to LTB_4, on average a 5-fold increase in plasmatic α-defensin levels was measured (Fig. 7*A*). Peak plasmatic α-defensin levels were reached 120–240 min after LTB_4 injection followed by a gradual decrease and return to baseline levels 24 h after LTB_4 injection.

The blood samples collected following the administration of LTB_4 to monkeys were analyzed for absolute circulating PMN numbers. Before LTB_4 injection ($t = -5$ min), the mean circulating PMN count was 1.5×10^6/ml on average (Fig. 7*B*). Two minutes after LTB_4 injection, a pronounced (>90%) drop in circulating PMN was recorded for all monkeys. This neutropenia was followed by a rapid increase in the total number of circulating PMN. In fact, 15 min after LTB_4 injection the PMN blood count was above baseline values for most monkeys. This neutrophilia, which reached its maximal level (mean of 4.6-fold over baseline) between 30 and 120 min gradually leveled off and was no longer significantly elevated 24 h after LTB_4 administration.

Ex vivo antimicrobial activity in plasma of monkeys treated with LTB_4

Having determined that i.v. LTB_4 triggered an increase of antimicrobial proteins (α-defensin) in the plasma of monkeys, we next studied the ex vivo antimicrobial activity of plasma samples. Plasma samples obtained from $t = -5$ (control), +120, and +240 min relative to the LTB_4 injections were used and tested for neutralizing efficiencies against virus and bacteria as described in *Materials and Methods*. When compared with the $t = -5$ min control plasma samples, those collected 120 and 240 min after injection of 50 μg/kg of LTB_4 exhibited a significantly higher HSV-1 killing activity (Fig. 7*C*). Our results also indicate that the plasma of monkeys obtained 120 and 240 min following LTB_4 injections possess significantly greater bactericidal activity than control plasma samples obtained 5 min before LTB_4 injections. Activities of monkey plasma injected with LTB_4 were also observed, albeit the effects against *S. aureus* were less pronounced than for *E. coli* or HSV-1.

Phamacokinetics of LTB_4

The pharmacokinetics of i.v. administered LTB_4 (50 μg/kg) was studied in 4 monkeys by measuring immunoreactive LTB_4 in plasma at various time points relative to the bolus injection. The results obtained (Fig. 7*D*) indicate that maximal plasmatic concentrations (C_{max}) were measured 2 min after LTB_4 injection, the first time point investigated. Plasmatic immunoreactive LTB_4 levels dropped rapidly to 10% of C_{max} at 15 min after LTB_4 injection. These results indicate that LTB_4 is rapidly eliminated from the circulation ($t_{1/2} < 15$ min) and that at time points where plasma samples show increased antimicrobial activities, the LTB_4 plasmatic levels were close to baseline values. It is worth noting that because the plasmatic LTB_4 levels measured by ELISA are likely overestimated because of interference by plasma components, the results are expressed in arbitrary units rather than absolute LTB_4 levels.

Discussion

In the present work we sought to extend our knowledge of the biological activities of LTB_4, in particular the efficiency of LTB_4 in triggering the release of antimicrobial molecules from isolated PMN in vitro as well as in vivo. LTB_4 is mostly known for its potent chemotactic activity toward various leukocytes. We have previously reported (29) that LTB_4 causes the in vitro and in vivo release of α-defensins from PMN isolated from healthy and HIV-1-infected individuals, but the effective antimicrobial potential of supernatants from LTB_4-activated PMN or of plasma from animals treated with LTB_4 was not investigated. We now extend these studies to show that the stimulation of isolated PMN with LTB_4 induces a rapid release of molecules that possess antiviral and antibacterial activities. These LTB_4-induced soluble factors destroyed the infectivity of enveloped viruses (HSV-1 and HIV-1) within minutes. Such rapid microbicidal effect could be an important feature considering that α-defensins have been reported to lose activity in the presence of serum or serum albumin (7). However, a detailed analysis of the inactivation of α-defensins in circulation has not been reported and the conclusions drawn from in vitro experiments may be misleading. In fact, our data suggest that plasma samples from monkeys treated with LTB_4 that contain increased levels of α-defensin show greater ex vivo antimicrobial activities relative to control plasma samples. These results suggest that the antimicrobial substances released in the circulation retain their activity (at least partly) for several minutes/hours, the time needed to isolate the plasma and perform the experiments. The exact nature of the agents responsible for the antimicrobial activities reported in the present study remains to be determined. The finding of α-defensins in the supernatants of LTB_4-activated PMN was expected given that defensins are major components of primary granules and that LTB_4 is known to induce PMN degranulation. However, considering the plethora of microbicidal substances present within PMN granules, the antimicrobial activity is likely multifactorial. In fact, mass spectroscopy analysis of proteins released by PMN following LTB_4 stimulation confirmed the presence of several other antimicrobial proteins, namely cathepsin G, neutrophil elastase, proteinase 3, lysozyme C, lactoferrin, and Cap-18/LL-37 in the PMN supernatant following LTB_4 stimulation.

To the best of our knowledge, this is the first study linking LTB_4, antimicrobial protein release from PMN, and demonstrated antimicrobial activity in vitro and in vivo. Interestingly, a recent work suggests that α-defensins can trigger the release of LTB_4 from alveolar macrophages (19), indicating that α-defensins and LTB_4, through positive feedback loops, can influence each other's formation and release. Even more recently, CC chemokines (RANTES, MIP-1α, and MIP-1β) were reported to induce PMN degranulation and α-defensin release (39). Considering that LTB_4 also induces the release of human MIP-1β under in vivo conditions (29) and that β-chemokines trigger the release of α-defensins, additive and possibly synergistic effects between LTB_4 and CC-chemokines on α-defensin release are expected.

Although human defensins were identified ~20 years ago, a renewed interest in these peptides occurred following a publication establishing a possible link between α-defensins and HIV-1-infected long-term nonprogressors (40). Numerous papers revisited and expanded upon the original paper describing anti-HIV-1 activity of α-defensins (41). The anti-HIV-1 activity of α-defensins is reported to be dual, the peptides acting directly as virucidal compounds and indirectly as modulators of transcription (42, 43). Interestingly, potential in vivo roles for α-defensins in the protection against HIV-1 infection were recently reported. In one study, breast milk α-defensin concentration was significantly associated with a decreased risk of intrapartum and postnatal HIV-1 transmissions (44). In a second study, an elevated HIV-1 burden in lymphoid follicles relative to extrafollicular lymphoid tissue could be correlated with

lower levels of naturally produced antiviral molecules, including α-defensins (45).

We demonstrated herein using monkeys and previously in a phase I clinical trial (29) that i.v. injection of LTB_4 result in increased plasmatic α-defensin levels. α-Defensin levels reached a maximum level 1–2 h after LTB_4 injection as measured by ELISA. For monkey α-defensin determinations, we made use of a commercial human α-defensin ELISA that cross-reacts with rhesus monkey and cynomolgus macaque α-defensins (Hycult Biotechnology). Although the sequence of the myeloid α-defensins of *M. fascicularis* are not yet known, they likely resemble closely those of humans, considering that rhesus monkey α-defensins share >85% identity with human α-defensins, with diverging amino acids representing highly conservative substitutions (46). Knowing this and the fact that the Abs used in the ELISA cross-react with human and macaque α-defensins, the standard curve was generated using recombinant human α-defensins. Although we have no direct evidence that enhanced plasmatic levels of α-defensins are the consequence of PMN degranulation, our in vitro studies suggested that this is likely to be the case. Indeed, in support of in vivo PMN activation by LTB_4, we have observed in vivo increased expression of cell surface CD11b (data not shown), a secretory granule membrane protein and PMN activation marker. Moreover, in response to LTB_4 administration, PMN rapidly disappeared from the circulation (transient neutropenia), which also reflected PMN activation by LTB_4. This neutropenia was followed by a neutrophilia that lasted 2–4 h, after which the circulating PMN counts returned to normal levels. Interestingly, α-defensin plasmatic levels correlated with the number of circulating PMN, suggesting a causal relationship between neutrophil mobilization and plasmatic α-defensin levels. Important differences exist however with regard to in vitro α-defensin release from LTB_4-stimulated PMN and enhanced plasma α-defensin levels following i.v. LTB_4 administration. In vitro, in the absence of cytochalasin B pretreatment the release of anti-microbial activity was not detectable and a slight increase in α-defensin release (1.5–2×) was observed at the highest LTB_4 concentration only (1 μM) (data not shown). Cytochalasin B and similar compounds disrupt the actin cytoskeleton, facilitating the release of cytoplasmic granules. Much like our results, the in vitro release of azurophilic granules that contain α-defensins from fMLP-stimulated PMN require the presence of an actin cytoskeleton-disrupting agent (47). Therefore, the question that arises is what could substitute for cytochalasin B priming in the in vivo setting? The adherence of the PMN to the endothelium is certainly to be considered as a priming event, as reported previously (48). In fact, PMN adherent to extracellular matrix proteins or endothelial cells respond to inflammatory mediators by releasing reactive oxygen intermediates and granule constituents (49–53). These responses are linked to the cytoskeleton reorganization that occurs when the PMN adheres to the extracellular matrix or endothelium (53–55). However, unlike in vitro α-defensin release, which occurs almost instantaneously following LTB_4 stimulation of primed PMN, the plasmatic in vivo α-defensin accumulation typically reaches maximal levels 2 h after LTB_4 injection. One explanation could be that the putative in vivo physiological priming triggered by the massive adhesion of PMN to vascular endothelium (following LTB_4 injection) occurs more slowly than under in vitro conditions. Alternatively, the in vivo plasmatic increase in α-defensins following LTB_4 injection may simply result from the transient increase in circulating neutrophils. In support of this, the in vivo kinetic of PMN accumulation coincides with the kinetic of α-defensin plasmatic levels. Furthermore, in vitro data suggest that spontaneous release of α-defensin from isolated PMN does occur in the absence of stimulation (data not shown) and that the α-defensin levels in supernatants is directly proportional to the PMN concentration. Therefore, the increase in the plasmatic α-defensin level following the injection of LTB_4 is likely the result of a combination of priming by adherence and an increase in circulating neutrophils. One striking observation from these animal studies is that the peak pharmacodynamic effects triggered by LTB_4 long outlasted the presence of plasmatic LTB_4. In fact, as observed previously (56), the elimination of LTB_4 from the circulation is very rapid, with >90% of the LTB_4 injected eliminated within 15 min. These results suggest that the biological effects triggered by LTB_4 are maintained for several hours.

Whether LTB_4 could be a useful immunostimulant for the treatment of infectious diseases remains to be investigated in clinical trials; numerous animal studies have already reported a beneficial effect of this eicosanoid models of infection (57–64). LTB_4 administration to humans has been documented (29, 65, 66), with pharmacodynamic effects (α-defensin and MIP-1β release (29) and PMN count variations (our unpublished data)) similar to what is reported in this study in monkeys without significant adverse events. Interestingly, low local levels of LTB_4 were reported in the lungs and brains of HIV-1-infected individuals afflicted with bacterial pneumonia and fungal encephalitis, respectively (67, 68). Furthermore, peripheral blood PMN (58, 69), monocytes (70), and alveolar macrophages (71) from HIV-1-infected subjects have a profound defect in their capacity to produce leukotrienes. This deficit translates into the improper in vitro killing of microbes but can be overcome by G-CSF administration to HIV-1-infected subjects and the concomitant restoration of leukotriene biosynthesis potential (58).

In conclusion, LTB_4 efficiently triggers, both under in vitro and in vivo conditions, the release of broad spectrum microbicides from PMN. Antimicrobial proteins, rather than low molecular mass antimicrobial agents such as hydrogen peroxide or NO, appear to account for the antimicrobial activity found in LTB_4-activated PMN supernatants. Further studies are needed to characterize the regulation of the release of these potent microbicides from PMN and their mechanisms of action as well as to determine the potential usefulness of LTB_4 as a therapeutic agent for the treatment or prophylaxis of infectious diseases.

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Louis Flamand and Pierre Borgeat are consultants for and hold stock in Innatis Pharma. Pierre Borgeat sits on the board of directors of Innatis Pharma.

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