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Leukotriene B<sub>4</sub> Induces Release of Antimicrobial Peptides in Lungs of Virally Infected Mice<sup>1</sup>

Éric Gaudreault and Jean Gosselin<sup>2</sup>

Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is a lipid mediator of inflammation that was recently shown to exert antiviral activities. In this study, we demonstrate that the release of antimicrobial proteins by neutrophils contribute to an early host defense against influenza virus infection in vitro as well as in vivo. Daily i.v. treatments with LTB<sub>4</sub> lead to a significant decrease in lung viral loads at day 5 postinfection in mice infected with influenza A virus compared with the placebo-treated group. This reduction in viral load was not present in mice deficient in the high-affinity LTB<sub>4</sub> receptor. Viral clearance in lungs was associated with up-regulated presence of antimicrobial peptides such as β-defensin-3, members of the mouse eosinophil-related RNase family, and the mouse cathelicidin-related antimicrobial peptide. Our results also indicate that neutrophils are important in the antiviral effect of LTB<sub>4</sub>. Viral loads in neutrophil-depleted mice were not diminished by LTB<sub>4</sub> administration, and a substantial reduction in the presence of murine cathelicidin-related antimicrobial peptide. Moreover, pretreatment of cell cultures with specific LTB<sub>4</sub> receptor antagonists clearly demonstrate the implication of the high-affinity LTB<sub>4</sub> receptor in the LTB<sub>4</sub>-mediated activity. Together, these results demonstrate the importance of neutrophils and the secretion of antimicrobial peptides during the early immune response mediated by LTB<sub>4</sub> against a viral pathogen.

<sup>1</sup>Abbreviations used in this paper: LTB<sub>4</sub>, leukotriene B<sub>4</sub>; CRAMP, cathelicidin-related antimicrobial peptide; EDN, eosinophil-derived neurotoxin; mEARs, murine eosinophil-related RNase family; MDCK, Madin-Darby canine kidney.

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eicosinophil-derived neurotoxin (EDN) has been detected on the surface of human neutrophils (30). EDN has known antiviral activities against respiratory viruses such as respiratory syncytial virus group B (31) and its related rodent counterpart, pneumonia virus of mice (32). EDN also has partial inhibitory activity against HIV-1 (33). In mice, EDN counterparts are RNases of the murine eosi-nophil-associated RNase family (mEARS), with mEARS possessing antiviral activity against pneumonia virus of mice (34).

In this study, we investigate the role of LTB4 in the control of infection by respiratory viruses. The viral pathogen chosen was human influenza virus due to its capability to efficiently infect mice. In addition, influenza infection is mostly controlled by neutrophil recruitment to the lung (35, 36), providing an interesting model of innate immunity.

The aim of this study was to determine which types of antimicrobial peptides are released by LTB4-treated cells leading to a reduction in viral load during in vitro and in vivo infection with influenza A virus and whether such an effect is mediated through BLT1R or BLT2R receptor.

Materials and Methods

Cell preparation and treatment

Neutrophils were obtained from peripheral blood of healthy medication-free volunteers after informed consent in accordance with an Internal Review Board-approved protocol. Briefly, peripheral blood leukocytes were enriched by dextran sedimentation, layered over a lymphocyte separation medium cushion, and centrifuged at 1400 rpm for 20 min. Mononuclear leukocytes were collected at the interface, whereas neutrophils were obtained from the pellet as described (37). Cell preparations were depleted of erythrocytes by osmotic shock and then washed and resuspended in HBSS supplemented with calcium until use. Purity of neutrophil preparations was >99%. No significant percentage of other types of granulocyte was detected in cell preparation as evaluated by cytomtery analysis. When applicable, cellular preparation could also be depleted of monocytes by adherence of PBMC on autologous serum-treated petri dishes.

Cell cultures were infected with influenza virus at 0.1 multiplicity of infection before a stimulation, 2 min later, with different doses of LTB4 (0.1, 1 or 10 nM; Johnson Matthey) for 48 h, without cell washing, to investigate direct virion inactivation mediated by LTB4 treatment. LTB4 was obtained as an ethanolic solution of the acid form and prepared by dilution of the ethanolic LTB4 in a saline solution 0.45% w/v NaCl containing 0.25% w/v dextrose. Saline solution without addition of LTB4 was diluted in NaCl, 0.45% w/v, containing dextrose, 0.25% w/v, before administration. Placebo treatment was referred to saline solution without LTB4, and lungs were harvested for viral load assessment.

Influenza A virus (strain A/Puerto Rico/8/34) and treated i.v. with 1000 ng/kg LTB4 (1000 ng/kg) for 1 h and sacrificed for lung extraction. Lungs were embedded in OCT compound. Sections of 5 μm were mounted in Superfrost-Plus slides for immunohistochemistry and hematoxylin staining. Immunohistochemistry was performed using an indirect method. Sections were treated with 0.3% hydrogen peroxide for 30 min. Sections were then blocked with 3% FBS for 30 min followed by incubation with isotypic or anti-CRAM Abs (provided by Dr. R. L. Gallo, University of California, San Diego, CA), anti-β-defensin-3 Abs (Alpha Diagnostic Int.), anti-IgG (HyCult Biotechnology), or anti-EDN (MBL Corp.) Abs (1 μg/ml) before LTB4 stimulation.

Viruses propagation and titration

Influenza A virus (strain A/Puerto Rico/8/34, H1N1) was propagated and titrated in cell cultures. Cells were infected with influenza virus at 0.1 multiplicity of infection before a stimulation, 2 min later, with different doses of LTB4 (0.1, 1 or 10 nM; Johnson Matthey) for 48 h, without cell washing, to investigate direct virion inactivation mediated by LTB4 treatment. LTB4 was obtained as an ethanolic solution of the acid form and prepared by dilution of the ethanolic LTB4 in a saline solution 0.45% w/v NaCl containing 0.25% w/v dextrose. Saline solution without addition of LTB4 was used as placebo. Cell-free supernatants were then harvested for viral load determination on Madin-Darby canine kidney (MDCK) cells. In some experiments, peripheral blood leukocytes or isolated neutrophils were pre-treated or not with the specific BLT1 antagonist U75302 (1 M) followed by treatment with cytochalasin D (10 μM) for 1 h in a humidified chamber. Sections were washed in PBS, incubated with biotinylated secondary Abs for 1 h at room temperature followed by incubation with HRP-streptavidin complex for 20 min, and visualized using diaminobenzidine substrate followed by counterstaining with hematoxylin.

Confocal microscopy

Lung tissues were prepared as described in Immunohistochemistry. Sections were incubated for 1 h with anti-CRAM Abs, washed three times, and incubated with anti-rabbit ALEXA 488-conjugated Abs for 1 h. Sections were then stained for 1 h with PE-conjugated anti-IgG (BD Biosciences), a specific marker of mature murine neutrophils. Fluorescence was visualized by confocal microscopy.

ELISA assay

Neutrophils (2 × 10⁶) were allowed to sediment for 1 h at 37°C in the presence or not of U75302 (1 μM) followed by treatment with cytochalasin B (10 μM) for 30 min at 37°C. Cytochalasin B was used as a priming agent for neutrophil degranulation and has no significant effect on cell activation (40, 41). After cytochalasin treatment, cells were stimulated or not with LTB4 for 30 min. Next, the cellular suspension was centrifuged at 4°C, and cell-free supernatant was collected and stored at −80°C until assayed for LL-37 (HyCult Biotechnology) or EDN (MBL Corp.) by ELISA following the manufacturer’s instructions.

Flow cytometric analysis

Neutrophils (2 × 10⁶ cells) were pretreated for 30 min with cytochalasin B (10 μM) followed by incubation with or without LTB4 (10 nM) for 1 h.
Cells were fixed for 15 min at room temperature with 2% paraformaldehyde and then incubated in the presence of anti-EDN Ab for 45 min. After three different washes with HBSS, goat anti-mouse FITC Ab was applied, and incubation for 45 min was performed. Cell surface expression of EDN was analyzed on 10,000 cells per sample using an EPICS XL apparatus (Beckman Coulter).

Statistical analyses

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

Results

Treatment with LTB$_4$ i.v. reduces influenza viral loads in mice

We first wanted to investigate the antiviral potential of LTB$_4$ in a mouse model of influenza infection. Although the BALB/c mouse strain is usually used for studies on viral infection of the lungs due to high viral replication levels and high viral titers on an extended period of time, we chose to use C57BL/6 mice. Infection with influenza virus of C57BL/6 mice leads to high viral titers in lungs of mice early during infection. In addition, we also chose C57BL/6 mice to facilitate comparison and interpretation of data obtained using BLT1R knockout mice generated on a C57BL/6 background.

Mice were infected intranasally with influenza A virus (strain A/PR/8/34) followed by daily i.v. treatment with LTB$_4$. Whereas C57BL/6 mice naturally control viral infection by reducing viral loads, administration of LTB$_4$ potentiated viral clearance (82% reduction in viral loads in lungs at day 5 postinfection), as opposed to mice treated with a placebo (Fig. 1). To verify the implication of the high-affinity LTB$_4$ receptor in such antiviral defense against influenza infection, we performed the same experiment using wild-type or BLT1R-deficient mice. Mice were infected intranasally with influenza virus and were treated daily by i.v. injection starting 24 h postinfection, followed by sacrifice and lung removal on day 5. Wild-type mice treated with LTB$_4$ showed a significant reduction in viral load as opposed to wild-type mice treated with a placebo (Fig. 2). On the other hand, no viral load reduction was observed in BLT1R$^{-/-}$ mice treated with LTB$_4$ compared with placebo treatment.

FIGURE 2. In vivo LTB$_4$ antiviral activity involves BLT1R activation. Female 5- to 6 wk-old C57BL/6 wild-type (WT) mice or BLT1R$^{-/-}$ mice (5 mice/group) were infected intranasally with 50 PFU of influenza A virus (strain A/PR/8/34). Mice were treated daily by i.v. injection with placebo or LTB$_4$ (1000 ng/kg) starting 24 h postinfection. Lungs were harvested at day 5 for viral load assessment by plaque assay. Results are expressed as the mean viral load ± SEM and are representative of two independent experiments. *, $p < 0.05$ compared with respective placebo-treated group.

FIGURE 3. LTB$_4$ treatment helps to restore normal lung architecture in influenza-infected mice. Female C57BL/6 mice (5–6 wk old; 5 mice/group) were infected intranasally with 50 PFU of influenza A virus (strain A/PR/8/34). Mice were treated daily by i.v. injection with placebo or LTB$_4$ (1000 ng/kg) starting 24 h postinfection. Lungs were harvested at day 5 postinfection and lung sections were stained with H&E. A, Example of alveolar structure; B, example of bronchiolar structure. Original magnification, ×100.

FIGURE 4. LTB$_4$-mediated up-regulation of β-defensin-3 in lung tissue of mice during viral infection. Female C57BL/6 wild-type (WT) mice or BLT1R$^{-/-}$ mice (5–6 wk old) were infected or not intranasally with 50 PFU of influenza A virus (strain A/PR/8/34). Twenty-four hours postinfection, mice were treated by i.v. administration of LTB$_4$ (1000 ng/kg; +LTB$_4$) or a placebo (−LTB$_4$) for 1 h before lung extraction. Immunohistochemistry was performed on lung tissue sections stained with isotype or anti-β-defensin-3 Abs as detailed in Material and Methods. Dark brown staining indicates β-defensin-3 expression. β-Defensin-3 expression seems to be detected in the epithelium of the bronchiolar airways. B, Example of bronchiolar structure. Original magnification, ×400.
knockout mice treated with placebo. These results clearly demonstrate that the antiviral activity of LTB4 against a respiratory viral pathogen occurs via the activation of the high-affinity BLT1 receptor.

Influenza infection is mostly associated with a massive lymphoid cell recruitment infiltrating the epithelium of lung tissue along with a decrease number of alveolar macrophages (42). Tissue changes are also observed during the course of infection with cellular exudates present in the luminal content of the airways as well as significant epithelial cell changes and necrosis. To evaluate whether LTB4 treatments could ameliorate tissue integrity in the lungs of influenza-infected mice, we infected mice with influenza followed by daily i.v. LTB4 treatments from days 1–4 postinfection. Mice were sacrificed at day 5 postinfection, and lungs were harvested and stained using H&E. As shown in Fig. 3, mice treated with LTB4 showed a significant cellular recruitment only to the lungs, probably of neutrophils to mount a potential immune response against an invading agent. When mice were infected with influenza and left untreated, massive leukocyte recruitment was observed in lung tissue. Moreover, tissue changes were also present in the epithelium as well as thickening of the alveolar wall.

When mice were treated with LTB4 after influenza infection, a more natural histological lung architecture was present, with less leukocyte infiltration as well as less plugging of the airways and thickening of the alveolar wall. These results demonstrate that not only does LTB4 reduce viral load in lung tissue of infected mice, but it also helps to restore a more normal lung architecture.

When mice were treated with LTB4 after influenza infection, we next investigated by which mechanism(s) LTB4 administration could potentiate viral clearance. We already demonstrated that LTB4 administration to healthy human subjects lead to secretion of defensins of the H9251 subtype by neutrophils (15). Because mice do not produce H9251-defensins, we tested for the presence of other antimicrobial peptides with antiviral potential following LTB4 treatment. Mice were infected or not with influenza virus and injected with placebo or LTB4 24 h postinfection. One hour after LTB4 treatment, mice were sacrificed, and lung tissues were harvested for detection of antimicrobial peptides. As shown in Fig. 4, a slight up-regulation in H9252-defensin-3 was observed following influenza infection probably due to natural immune activation in the lungs.
A slight up-regulation in β-defensin-3 was also observed in mice that were treated with LTB₄ without viral infection (data not shown). However, when wild-type mice were infected with influenza followed by treatment with LTB₄, a strong up-regulation in β-defensin-3 was also accompanied by a reduction in viral loads in lungs of infected mice treated with LTB₄. On the other hand, up-regulation of β-defensin-3 was not observed in lungs of infected BLT₁R−/− mice, demonstrating the requirement for BLT₁R activation for such engagement of the innate immune response. A similar pattern of expression was observed for other peptides bearing antiviral potential such as mEARS (Fig. 5) and CRAMP proteins (Fig. 6). CRAMP proteins are mostly produced by neutrophils and to a much lesser extent by epithelial cells. Because immunohistochemistry using anti-CRAMP Abs could not identify with absolute certainty that CRAMP-positive cells were in fact neutrophils, we next performed confocal microscopic examination using the selective marker of murine neutrophil, Gr-1/Ly6G. As shown in Fig. 7, intracellular CRAMP protein colocalizes with Gr-1/Ly6G-positive neutrophils (yellow color on merged panels) that migrated in lung tissue following influenza infection. This observation clarifies the fact that murine neutrophils do produce CRAMP protein after LTB₄ administration during viral infection. It is interesting to note the presence of extracellular staining of CRAMP protein in lung tissue that might be the result of neutrophil degranulation following stimulation by LTB₄. This extracellular detection of CRAMP protein seems to be associated with interstitial tissue area where neutrophils migrate after viral threat infection. Moreover, more CRAMP⁺Gr-1/Ly6G⁺ neutrophils were present in lung tissue of infected wild-type mice treated with LTB₄ as opposed to healthy mice or infected mice treated with placebo. All the results presented above demonstrate that LTB₄ administration leads to antimicrobial peptide secretion in lungs of mice, a process that may help control influenza infection.

Neutrophils contribute to the antiviral activity of LTB₄ in vivo

We previously hypothesized that LTB₄ might target neutrophils to reduce viral presence in infected tissue (15). To investigate the effect of LTB₄ on neutrophil mobilization in mice as well as the role of neutrophils in viral clearance, we injected Abs directed against Ly6G/Gr-1 Ag i.v. into mice to deplete peripheral neutrophils. As shown in Table I, following LTB₄ injection, a substantial reduction in peripheral blood neutrophils was observed, suggesting

<table>
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<tr>
<th>Treatment</th>
<th>% Ly6G/Gr-1⁺ Neutrophils in Blood</th>
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<tbody>
<tr>
<td>Placebo</td>
<td>15.65 ± 2.17</td>
</tr>
<tr>
<td>LTB₄</td>
<td>6.29 ± 1.94⁺</td>
</tr>
<tr>
<td>Influenza</td>
<td>17.70 ± 5.27</td>
</tr>
<tr>
<td>Influenza + LTB₄</td>
<td>29.42 ± 6.46⁺</td>
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* Mice (6/group) were depleted of neutrophils by i.p. injection of 250 μg of anti-Ly6G/Gr-1 Abs at days −1 and 2 postinfection. Infection was performed intranasally with influenza virus (50 PFU) followed by daily i.v. injection with LTB₄ (1000 ng/kg) from day 1 to day 4 postinfection. Mice were sacrificed on day 5 postinfection.  

*p < 0.05 compared with placebo-treated mice. Percentage of blood neutrophils was evaluated by flow cytometry.
that neutrophils leave the blood circulation after LTB₄ administration. As expected, no neutrophil could be detected in the bloodstream of mice injected with anti-Ly6G/Gr-1 Abs. Although blood neutrophil counts were not affected by influenza infection, the number of circulating neutrophils increased when mice were infected with influenza virus and treated with LTB₄. This fact is intriguing, given that neither LTB₄ administration nor influenza infection seemed to promote hematopoiesis (E. Gaudreault and J. Gosselin, personal observation).

As expected, daily administration of LTB₄ maintains recruitment of neutrophils in lung tissues even after 4 days of treatment, as compared with the placebo group (Table II). A more substantial mobilization of neutrophils in lungs of mice was also observed at 1 h after LTB₄ administration (data not shown). The number of neutrophils in lungs of mice infected with influenza virus was also found to increase, a process that may reflect a natural host defense against virus infection, as previously suggested by others (45). In line with these results, a massive neutrophil recruitment was also observed in lung tissues of mice infected with influenza virus and treated with LTB₄ as opposed to all other experimental groups of mice (Table II). Overall, this set of experiments suggests that LTB₄ administration to mice infected or not with influenza virus potentiates migration of neutrophils from peripheral blood to the lungs, a mechanism that may contribute to enhance viral clearance.

### Table II. Percentage of lung neutrophils in mice depleted with anti-Ly6G/Gr-1 Abs

<table>
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<tr>
<th>Treatment</th>
<th>% Ly6G/Gr-1⁺ Neutrophils in Lungs</th>
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<tbody>
<tr>
<td></td>
<td>Nondepleted Mice</td>
</tr>
<tr>
<td>Placebo</td>
<td>5.76 ± 0.34</td>
</tr>
<tr>
<td>LTB₄</td>
<td>10.44 ± 2.13b</td>
</tr>
<tr>
<td>Influenza</td>
<td>15.40 ± 3.52b</td>
</tr>
<tr>
<td>Influenza + LTB₄</td>
<td>26.94 ± 4.93b</td>
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</tbody>
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* Mice (6/group) were depleted of neutrophils by i.p. injection of 250 μg of anti-Ly6G/Gr-1 Abs at days −1 and 2 postinfection. Infection was performed intranasally with influenza virus (50 PFU) followed by daily i.v. injection with LTB₄ (1000 ng/kg) from day 1 to day 4 postinfection. Mice were sacrificed on day 5 postinfection.

b p <0.05 compared with placebo-treated mice. Percentage of lung neutrophils was evaluated by flow cytometry.

FIGURE 8. Neutrophils contribute to the antiviral effect of LTB₄ in vivo. A, Female C57BL/6 wild-type mice (5–6 wk old; 6 mice/group) were injected i.p. with 250 μg of anti-Ly6G/Gr-1 Abs at days −1 and + 2 postinfection. Mice were infected intranasally with 50 PFU of influenza A virus (strain A/PR/8/34) and treated daily by i.v. injection with placebo or LTB₄ starting 24 h postinfection. Lungs were harvested at day 5 postinfection for viral load assessment by plaque assay. Results are expressed as the mean viral load ± SEM. *, p < 0.05 compared with placebo-treated mice. B, Immunohistochemistry was performed on lung tissue sections from mice in A and stained with isotype (left), or with CRAMP, anti-β-defensin-3 or mEARs Abs (right). Dark brown staining indicates the presence of peptides. Arrows identify examples of positive cells for respective peptides. B, Example of bronchial structure. Original magnification, ×400.
significantly different compared with untreated cell preparations. LTBA at reducing lung influenza viral load in absence of neutrophils (LY255283, 1 μM) for 1 h before infection. Values are means ± SEM from three independent experiments done in triplicate. ∗, p < 0.05 indicates results significantly different compared with untreated cell preparations.

To further provide an in vivo confirmation of the link between neutrophil recruitment to the lung, influenza viral clearance, and secretion of antimicrobial peptides, we measured influenza titers in the lungs of mice depleted of neutrophils and performed lung immunoassaying for the presence of antimicrobial peptides following LTBA administration. As shown in Fig. 8A, when mice were injected with isotypic Abs and treated with LTBA after influenza infection, a significant decrease in lung viral titer was observed. On the other hand, when mice were depleted of their neutrophils and treated with a placebo, the lung viral titers were up-regulated by ~5-fold as opposed to mice treated with isotypic Ab. Moreover, LTBA administration was ineffective in reducing lung viral titers, showing the importance of neutrophils in the lung for LTBA-mediated antiviral activity. As expected, lung tissues of infected mice injected with isotypic Abs were positive for CRAMP, β-defensin-3, and mEARS staining after LTBA administration (Fig. 8B, left). Moreover, nondepleted uninfected mice that were given LTBA also showed positive staining for CRAMP, β-defensin-3, and mEARS proteins, albeit to a lesser extent than infected mice treated with LTBA. In infected mice injected with anti-Ly6G/Gr-1, absence of CRAMP and mEARS as well as a slight reduction in β-defensin-3 staining were observed after LTBA administration (Fig. 8B, right). These results demonstrate that the inability of LTBA at reducing lung influenza viral load in absence of neutrophils might be associated with the reduction or absence of antimicrobial peptide secretion.

**LTBA induces antiviral activity in human neutrophils**

We next wanted to investigate whether the antiviral potential of LTBA during influenza virus infection in mice was also present following in vitro infection of human leukocytes with influenza. For that matter, peripheral blood leukocytes were isolated and infected with influenza virus followed by a treatment with LTBA. As shown in Fig. 9, LTBA administration (1, 10, and 100 nM) leads to a statistically significant reduction of influenza viral load as opposed to cells only infected with the virus. When neutrophils were depleted from the cellular preparation, no significant reduction in viral load was observed after treatment with 10 nM LTBA. This was not the case when monocytes were depleted from the cellular preparation (data not shown). Such results support, once again, the fact that neutrophils are the major cellular players in LTBA-mediated antiviral activity. Although under natural conditions influenza virus targets preferentially lung epithelial cells for replication, we chose to use a model of peripheral blood leukocyte infection to clearly demonstrate the implication of neutrophils in LTBA-mediated antiviral activity. As also shown in Fig. 9, when leukocytes were pretreated with a specific BLT1 antagonist (U75302; Ref. 45), the reduction in viral load was not observed following treatment with LTBA. Pretreatment with the specific BLT2 antagonist, LY255283, did not succeed in abrogating LTBA antiviral activity. These results support the antiviral effect of LTBA in vitro against influenza virus infection of human cells via a neutrophil-dependent mechanism which involves BLT1R receptor.

**LTBA-mediated antiviral activity of human neutrophils involves the secretion of antimicrobial peptides**

Cathelicidins are a family of antimicrobial proteins found in peroxidase-negative granules of neutrophils. In human neutrophils, hCAP-18 is the only cathelicidin detected. Cleavage of hCAP-18 by proteinase 3 gives rise to LL-37 in the exocytosed material from neutrophils (24). EDN is a member of the RNase A superfamily and was found to be produced by neutrophils. EDN has antiviral activity in vitro, thus making EDN an antimicrobial mediator. Because neutrophil granule secretion seems to be an important strategy in the LTBA-mediated neutrophil antiviral activity, we next investigated whether LTBA treatment might induce the production of LL-37 and EDN during viral infection. First, we observed that LTBA induces the release of LL-37 (Fig. 10A) and EDN (Fig. 10B) in a dose-dependent manner by neutrophils reaching peak secretion at a concentration of 100 nM LTBA. Influenza virus itself did not promote neutrophil secretion of LL-37 and EDN in such in vitro setting (data not shown) as opposed to other viruses such as CMV known to induce secretion of such peptides by neutrophils (41). As previously mentioned, β-defensin-3 expression was up-regulated in mouse lung tissue following LTBA administration. However, human neutrophils do not seem to produce β-defensin-3. Whereas β-defensins have been isolated from leukocytes and epithelial tissues, human neutrophils express only low levels of β-defensin-4 (HBD-4), which displays a selective spectrum of antibacterial activity (46). We could not detect up-regulation in HBD-4 transcription following stimulation of human neutrophils with LTBA or influenza virus (data not shown). This observation suggests that the release of HBD-4 may be regulated by different stimuli and different signal pathways not activated by LTBA or viral
infection. The release of EDN molecules by neutrophils following LTB4 is of particular interest because, although the presence of EDN in neutrophils has been previously identified (29), its release from neutrophils following stimulation by LTB4 or other physiological agonists has not yet been reported. Similarly, the stimulation of neutrophils with LTB4 for 1 h also led to a significantly higher number of membrane-bound EDN molecules at the cellular surface of neutrophils (Fig. 10B, inset).

It seems likely that the release of H9251-defensins, LL-37, and EDN by neutrophils is an important event activated by LTB4 to counter influenza virus infection. To further support this assumption, we performed another set of experiments. Peripheral blood leukocytes were pretreated with a combination of neutralizing Abs directed against H9251-defensins, LL-37, and EDN before influenza infection and to treatment with LTB4 (10 nM). As shown in Fig. 10C, the effect of LTB4 in reducing viral loads was significantly affected by the presence of neutralizing Abs in cell cultures, indicating that LTB4 can mediate its antiviral effect through the release of H9251-defensins, LL-37, and EDN. However, because incubation of leukocytes with all three types of neutralizing Abs did not completely abrogate the effect of LTB4 on the viral load, we must consider that other mediators such as superoxide anion and/or the release of primary granules containing myeloperoxidase can also be activated in LTB4-treated leukocytes to reduce the number of viral particles.

We can now postulate that the secretion of the antimicrobial peptides defensins, EDN, and LL-37 (or their murine counterparts) may represent a mechanism triggered by LTB4 to counter viral spread.

Discussion
In this study, we provide biological mechanisms implicated in LTB4-mediated antiviral activity against in vivo as well as in vitro infection with influenza virus. We also demonstrate the potential implication of neutrophil secretion of antimicrobial peptides such as β-defensins, mEARS, and CRAMP in a mouse model of infection and human LL-37 and EDN in such activity. These LTB4-mediated neutrophil activities also involve the high-affinity LTB4 receptor, BLT1.

This study clearly shows that administration of leukotriene B4 in mice infected with influenza virus helps controlling viral infection via the triggering of BLT1R receptor. The antiviral action mediated by LTB4 seems to involve the secretion of antimicrobial peptides in lung tissue. We demonstrated that neutrophils leave the bloodstream to mobilize in the infected tissue (in this case the lung) following LTB4 administration. These neutrophils are believed to secrete peptides known to possess antiviral properties such as defensins, CRAMP, and mEARS to control and clear influenza infection.

Degranulation is a major biological activity of human neutrophils that encounter foreign microorganisms. This degranulation can be induced by LTB4 present at the site of infection (47, 48). Neutrophil granules such as azurophil/primary, secondary, and secretory granules contain different molecules known to possess antiviral properties. Defensins are cationic peptides that possess such antiviral activities. Already in 1986, Daher et al. (17) observed that the human β-defensin HNP-1 was able to directly inactivate
different viruses such as CMV, HSV types 1 and 2, vesicular stomatitis virus, and influenza virus strain A/WSN, probably by impairing the ability of such viruses to infect cells. During clinical trials with healthy human subjects, we also demonstrated that neutrophil HNPs are secreted by healthy patients following i.v. LTBA administration (14). Also, neutrophils from HIV-positive patients do retain the capability of secreting HNPs after in vitro stimulation with LTBA. In the present study, because mice do not produce defensins of the α subtypes, we evaluated the secretion of defensins of the β subfamily following LTBA administration. In this regard, β-defensin-3 is known to possess antiviral potential against multiple viruses such as HSV-1 (49), vaccinia virus (50), and HIV-1 (31). We observed an up-regulated expression of β-defensin-3 in lung tissue of influenza-infected mice treated with LTBA. Murine β-defensin-3 is already known to be inducible in epithelia of different organs following Gram-positive bacterial infection (51). Similarly, our results show that production of β-defensin-3 was induced in airways epithelial cells, but during the course of a viral infection. Moreover, β-defensin-3 induction was potentiated following LTBA administration. On the other hand, human neutrophils do not produce β-defensin-3 (as reviewed in Ref. 52), and to our knowledge murine β-defensin-3 has not been associated to neutrophils. It is therefore not surprising that the up-regulation in β-defensin-3 in lung tissue of infected mice treated with LTBA was also present in mice depleted of their neutrophils. In this case, LTBA would seem to act directly on epithelial, endothelial, or fibroblastic cells leading to secretion of such peptides. The fact that BLT1 mRNA is detectable in lung tissue of mice (43) and that human bronchial fibroblast cells (53), endothelial cells (44), and cells of the alveolar wall (54) express BLT1 at their surface further reinforce this assumption.

CRAMP protein staining was also up-regulated in lung tissue of mice following LTBA administration, and most intracellular CRAMP was present in neutrophils of the interstitial tissue. In addition, we provide evidence that LTBA does stimulate secretion of human LL-37 in a dose-dependent manner in vitro. Given that LL-37 exerts antiviral activities but also acts as a chemotactic agent for neutrophils, monocytes, and T cells (55, 56), we can postulate that LTBA administration provides a link between innate and adaptive immunity by stimulating neutrophils to secrete LL-37 that has the ability to recruit and stimulate cells of the adaptive immunity at the site of viral infection. Thus, we must consider that the release of LL-37 by LTBA-stimulated neutrophils should participate in the elimination of viral particles in vivo. The fact that more CRAMP peptide was secreted over a period of 24 h after LTBA administration happened daily following drug administration, and this accumulation of antimicrobial peptides in mouse lungs over time culminated in viral clearance. This hypothesis is supported by the fact that LTBA treatment seemed more beneficial after four administrations (day 5 postinfection) as opposed to two administrations (day 3 postinfection). At this moment, it is not clear why LTBA treatments were less successful at clearing influenza infection 7 days postinfection as opposed to 5 days. However, a probable explanation lies in the fact that granular pools of antimicrobial peptides in lung tissue neutrophils might be exhausted after repeated administrations of LTBA. This hypothesis is now under investigation. Moreover, because BLT1-mediated neutrophil degranulation involves the activation of the Src family kinase member Yes (59) and PI3K (60), it would be of great interest to further study the intracellular events implicated in BLT1-mediated antiviral neutrophil granule secretion.

To correlate results obtained with a mouse model to humans, we performed in vitro infection of peripheral blood leukocytes. Such population contains neutrophils that seem to be involved in LTBA-stimulated antiviral activity, given that neutrophil depletion abrogated such antiviral mechanism. This report also shows that human neutrophils do, in a dose-dependent manner, secrete LL-37 and EDN following LTBA stimulation. Such secretion, accompanied with α-defensin secretion, seem to be an important antiviral mechanism used by LTBA-stimulated neutrophils in the elimination of influenza virions. This fact was supported by the use of neutralizing Ab directed against such antimicrobial peptides.

In this study, we demonstrate a BLT1-dependent neutrophil antiviral activity directed against influenza virus following administration of LTBA. This neutrophil-mediated antiviral response implies the secretion of granules containing different peptides bearing antiviral activities such as α-defensin peptides, cathelicidins, and EDN. LTBA, by orchestrating a very complex antiviral immune response, can now be seen as a potential therapeutic agent for the treatment of a diversity of viral infections.

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