Leukotriene B_4 licenses inflammasome activation to enhance skin host defense

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The initial production of inflammatory mediators dictates host defense as well as tissue injury. Inflammasome activation is a constituent of the inflammatory response by recognizing pathogen and host-derived products and eliciting the production of IL-1β and IL-18 in addition to inducing a type of inflammatory cell death termed "pyroptosis." Leukotriene B_4 (LTB_4) is a lipid mediator produced quickly (seconds to minutes) by phagocytes and induces chemotaxis, increases cytokine/chemokine production, and enhances antimicrobial effector functions. Whether LTB_4 directly activates the inflammasome remains to be determined. Our data show that endogenously produced LTB_4 is required for the expression of pro-IL-1β and enhances inflammasome assembly in vivo and in vitro. Furthermore, LTB_4-mediated Bruton's tyrosine kinase (BTK) activation is required for inflammasome assembly in vivo as well for IL-1β–enhanced skin host defense. Together, these data unveil a new role for LTB_4 in enhancing the expression and assembly of inflammasome components and suggest that while blocking LTB_4 actions could be a promising therapeutic strategy to prevent inflammasome-mediated diseases, exogenous LTB_4 can be used as an adjuvant to boost inflammasome-dependent host defense.

inflammasome | leukotriene | skin | innate immunity

Upon infection, a fast and highly synchronized inflammatory response is mounted to restrict microbial growth and eventually eliminate the pathogen. Therefore, studies focusing on early events could unmask new players involved in the generation and magnitude of host defense. Inflammasomes are multiprotein intracellular platforms that detect both pathogen and host-derived products and induce the inflammatory response. The proteins that form the inflammasome consist of upstream sensors that belong to the nucleotide-binding oligomerization domain-like receptor (NLR) family, the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and the downstream effector caspase-1. There are different NLR proteins, including NLRP1, 2, 3, 6, 7, and NLRC4, and absent in melanoma 2 (AIM2) (1, 2). The different NLRs recognize both distinct and overlapping stressors to elicit the maturation and secretion of the inflammatory cytokines IL-1β and IL-18. Upon cell stimulation with either pathogen- or damage-associated molecular patterns (PAMPs and DAMPs, respectively), the inflammasome is activated in two sequential steps: 1) transcription of the long forms (procytokines) of IL-1β and IL-18; and 2) the assembly of the inflammasome complex ASC/NLRP and procaspase-1, followed by autocatalytic cleavage of caspase-1, and processing and secretion of mature IL-1β and IL-18, as well as the production of lipid mediators, such as prostaglandins and leukotriene B_4 (LTB_4) (3–5). The secretion of these cytokines is followed by a form of programmed cell death called pyroptosis that releases DAMPs and further amplifies the inflammatory response. Increased production of IL-1β and IL-18, along with DAMPs generation, have been heavily implicated in the pathogenesis of a myriad of inflammatory diseases and facilitates antimicrobial activities (1, 6).

Staphylococcus aureus skin infection is controlled by the synchronized actions of structural cells (keratinocytes) and skin phagocytes. Inflammasome-dependent IL-1β production is required for neutrophil recruitment, abscess formation, and bacterial clearance (7, 8). The mechanisms underlying inflammasome activation and IL-1β production during skin infection is not well understood.

LTB_4 is a bioactive lipid mediator that is quickly produced by phagocytes, such as macrophages and neutrophils. LTB_4 synthesis involves several rate-limiting steps that include activation of phospholipase A_2 (PLA_2) and arachidonic acid (AA) release

Significance

Production of IL-1β is an essential component of the inflammatory response and host defense. IL-1β secretion is dependent on the activation of an intracellular platform termed inflammasome. The initial inflammatory signals that drive inflammasome activation remains elusive. Here, we show that the bioactive lipid leukotriene B_4 enhances both transcriptional and posttranscriptional programs that activate the inflammasome in vivo and in vitro. We identified critical signaling programs required for inflammasome assembly, IL-1β secretion, and its consequences in skin host defense. Our data also suggest that the prevention of LTB_4 actions might be an important therapeutic strategy to prevent IL-1β–dependent inflammatory diseases by inhibiting both first and second signals necessary for inflammasome activation.

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The authors declare no competing interest.

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from phospholipids in cellular membranes. Activation of 5-lipoxygenase (5-LO) and 5-LO activation protein together metabolize AA to LTA_4, which is converted to LTB_4 by LTA_4 hydrolase (LTA_4H) (9). LTB_4 binds to two different G protein-coupled receptors (GPCR): a high-affinity receptor (BLT1) and a low-affinity receptor (BLT2) (10). LTB_4 via BLT1 enhances inflammatory response by increasing phagocyte chemotaxis and activating transcription factors required to produce inflammatory cytokines. We and others have shown that LTB_4 enhances pattern recognition response (PRR)-mediated responses by increasing the expression of the TIR adaptor MyD88 and the transcription factors NF-κB, AP-1, and PU.1 (11–14). Also, LTB_4 is required for the control of a myriad of pathogens once this mediator enhances microbial clearance by controlling the generation of reactive oxygen and nitrogen species and antimicrobial peptides in vivo (15–18). We have also shown that the treatment of mice with a topical ointment containing LTB_4 enhances *S. aureus* clearance from the skin, production of inflammatory mediators, and abscess formation (19). Whether LTB_4 enhances skin host defense directly or indirectly increases the production of proinflammatory mediators remains to be determined. Also, whether LTB_4 influences inflammasome activation is poorly understood. It has been shown that LTB_4-mediated arthritis severity and increased leishmanicidal activity is impaired in $ASC^{-/-}$ and $NLRP3^{-/-}$ mice (20, 21). However, the mechanisms underlying LTB_4-mediated inflammasome activation and whether LTB_4 is a component of the IL-1β–mediated skin host defense is unknown. Here, using epistatic and gain of function approaches, we demonstrate that LTB_4 is required for both first and second signals necessary for inflammasome activation and identified critical signaling programs required for inflammasome assembly, IL-1β secretion, and its consequences in skin host defense.

Methods

Animals. Mice were maintained according to NIH guidelines for using experimental animals with the approval of the Indiana University (protocol no.10500) and Vanderbilt University Medical Center (protocol no. M1600154) Animal Care and Use Committees. Experiments were performed following the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the US Animal Welfare Act. Eighteen-week-old female or male $BLT1^{-/-}$ (B6.129S4-*Ltb4r1*^{tm1Adl}/J (22), LysMcre, MMDTR, and strain-matched wild-type (WT) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Enhanced green fluorescent protein-LysM mice were donated by Nadia Carlesso, City of Hope, Duarte, CA, pIL1DsRed mice were donated by Akiko Takashima, University of Toledo, Toledo, OH (23), and $IL1R^{-/-}$ mice were donated by James Cassat, Vanderbilt University Medical Center, Nashville, TN.

Primary Cell Isolation and Culture. Murine resident peritoneal macrophages were isolated using ice-cold phosphate-buffered saline (PBS) as previously described (14, 24). To isolate neutrophils, bone marrow from both tibias and femurs were flushed with PBS (19), and neutrophils were negatively isolated using a MACSxpress Neutrophil Isolation Kit, following the manufacturer's instructions (Miltenyi Biotec, Sunnyvale, CA). The media used to culture primary cells was Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum (FBS), 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1× antibiotic/antimycotic (ThermoFisher Scientific, Waltham, MA).

Human Phagocyte Isolation and Culture. Samples were collected only from healthy donors with no significant medical conditions, aged 18–40 y at Hemoba (Fundação de Hematologia e Homoterapia do Estado da Bahia), Bahia/Brazil. Peripheral blood mononuclear cells collection and informed consent were approved by the Institutional Review Board at the Gonçalo Moniz Institute (Oswaldo Cruz Foundation-IGM-FIOCRUZ, Salvador, Bahia-Brazil). All of the blood samples were deidentified before use and the consent forms for the use of the samples also were kindly provided by the donors prior to performing the experiments. Peripheral blood polymorphonuclear (PMN) cells were isolated by centrifugation using Polymorphprep medium according to the manufacturer's instructions (Axis-Shield, Dundee, UK). Monolayers were harvested and washed with PBS at 4 °C, 400 × *g*. Cells were cultured in Roswell Park Memorial Institute medium 1640, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin G, and 100 mg/mL streptomycin (all from ThermoFisher Scientific) at 37 °C and 5% CO_2. PMNs were cultured with 100 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) for 2 h, washed with PBS, and challenged with 10 nM LTB_4. Culture supernatants were harvested to measure IL-1β release and active caspase-1 by enzyme-linked immunosorbent assay (ELISA) as described below.

Inflammasome Activation. Macrophages and neutrophils were challenged with 100 ng/mL LPS for 3 h, followed by treatment with nigericin 1 μM, monosodium urate crystal (MSU; 10 μM), flagellin 20 μg/mL, and Poly (dA:dT)/LyoVec 2 μg/mL for 1 h. To assess the role of LTB_4 in amplifying inflammasome activity, cells were pretreated with 10 nM LTB_4 or 10 μM BLT1 antagonist U-75302 5 min before the addition of the inflammasome activators. The direct effect of LTB_4 in inflammasome activation was studied in cells treated with LPS as above, followed by LTB_4 for 3 h.

Methicillin-Resistant *S. aureus* Skin Infection and Topical Ointment Treatment. Mice were infected with methicillin-resistant *S. aureus* (MRSA) USA300 LAC strain (~5×10^6 colony-forming units [cfu]) subcutaneously (s.c.) in 50 μL of PBS as previously shown (25). Lesion and abscess sizes were monitored and determined by affected areas calculated using a standard formula for the area: ($A = [\pi/2] \times l \times w$) (26). The final concentrations of the ointments per animal were as follows: LTB_4 (33.7 ng–3.37×10^{-6}%), U-75302 (0.001%), all in 1 g of petroleum jelly (vehicle control). The ointment treatments were applied to the infected area with a clean cotton swab. Mice were treated once a day throughout infection (ranging from 6 h to 9 d).

Skin Biopsies and Bacterial Load. Punch biopsies (8 mm) from noninfected (naive) or infected skin were harvested at different time points and used for determining bacterial counts, cytokine production, RNA extraction, cell isolation, histological analyses, and proximity ligation assay (PLA) staining (27). For bacterial load, skin biopsies were collected on day 1 postinfection, processed, and homogenized in tryptic soy broth medium. Serial dilutions were plated on tryptic soy agar (TSA) plates, and colonies were incubated at 37 °C, 5% CO_2, and counted after 18 h. Bacterial burdens were normalized to tissue weight and calculated by the following equation: ([cfu/mL plated]*[dilution factor])/(tissue weight in mg). Bacterial burden in the skin is represented as cfu/mg tissue (25).

Quantitative Real-Time PCR. Total RNA was isolated from cultured macrophages or skin biopsies using lysis buffer (RLT; Qiagen, Redwood City, CA). The RT^2 First Strand Kit reverse transcription system (Qiagen) was used for complementary DNA synthesis, and quantitative PCR (qPCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Gene-specific primers were purchased from Integrated DNA Technologies (Coralville, IA). Relative expression was calculated as previously described (28).

Immunoblotting. Western blots were performed, as previously described (12). Briefly, cell supernatant and cell lysate were collected, and proteins were precipitated using trichloroacetic acid. Cell lysate and supernatant were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with commercially available primary antibodies against caspase-1, IL-1β, and ASC (all at 1:1,000; Abcam, Cambridge, UK) phosphorylated BTK (Tyr223), total BTK (all at 1:1,000; Cell Signaling Technology, Danvers, MA) or β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO). Densitometric analysis was performed as described previously (12).

ELISAs. Skin biopsy sections were homogenized with a pestle in TNE cell lysis buffer containing 1× protease inhibitor (Sigma-Aldrich) and centrifuged to pellet the cellular debris. Cytokine levels detected in the skin were normalized to tissue weight and represented by ng/mg tissue. The supernatant of cultured macrophages or skin lysate from WT or $BLT1^{-/-}$ mice were also used to detect IL-1β, tumor necrosis factor (TNF)-α, or caspase-1 (Biolegend Inc., San Diego, CA).

Confocal Microscopy and PLA. PLA was performed using a Duolink PLA kit (Sigma-Aldrich) in skin sections of naive and infected WT and $BLT1^{-/-}$ mice or in vitro in peritoneal macrophages, and bone marrow neutrophils cultured in 8 well chambered cell culture slides (Corning Inc., New York, NY). In vitro, phagocytes were challenged with 100 ng/mL LPS or GFP-MRSA at MOI 50:1 for 3 h and pretreated with 10 μM BLT1 antagonist U-75302 or 10 nM LTB_4 for 15 min, followed by inflammasome activation (10 μM MSU or 1 μM

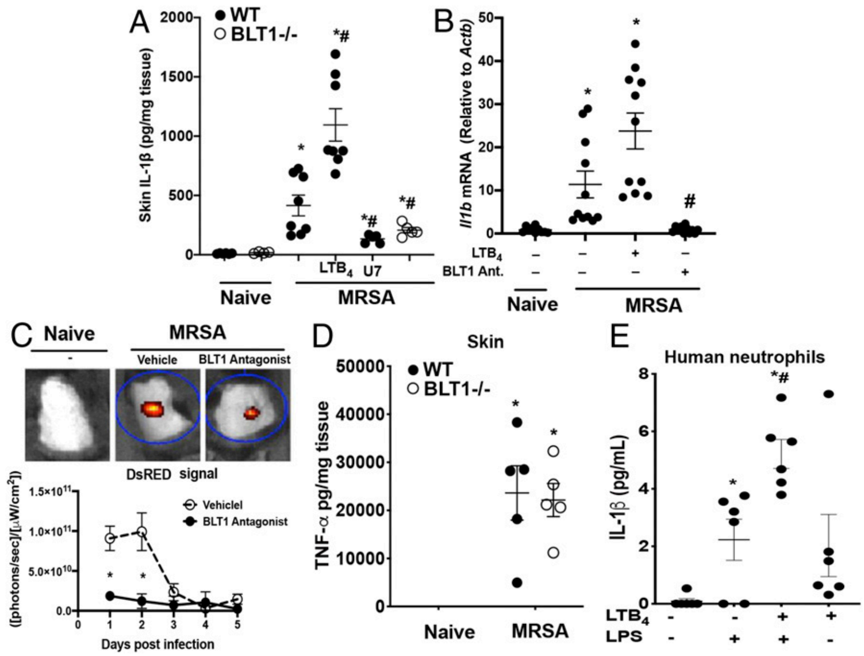

Fig. 1. LTB_4 is required for IL-1β production. (*A*) Quantification of IL-1β and (*B*) *Il1b* mRNA expression in the skin of C57BL/6, $BLT1^{-/-}$, C57BL/6 treated with a topical ointment containing LTB_4 or the BLT1 antagonist U-75302 24 h after MRSA infection. Data are mean ± SEM of 5–11 mice from, at least, two independent experiments. *$P < 0.05$ versus naive WT mice. #$P < 0.05$ versus infected WT mice. (*C*) *Top*, IVIS scanning of IL1DsRed mice infected and treated with a topical ointment containing BLT1 antagonist U-75032 or vehicle control. *Bottom*, quantification of photon/s of IL1DsRED overtime from mice treated daily or not with the BLT1 antagonist U-75302. Data are mean ± SEM of 6–10 mice/group from three independent experiments. *$P < 0.05$ versus pIL1DsRed mice treated with vehicle control. (*D*) TNF-α quantification in a skin biopsy from WT or $BLT1^{-/-}$ mice after 24-h MRSA infection. *$P < 0.05$ versus naive WT or $BLT1^{-/-}$ mice. (*E*) IL-1β quantification from human neutrophils isolated from healthy blood donors treated with LTB_4 and LPS. Data are the mean ± SEM of six different healthy donors performed in triplicate. *$P < 0.05$ versus naive. #$P < 0.05$ versus LPS.

nigericin) for 1 h. For in vivo PLA detection, WT mice (treated or not with an ointment containing LTB_4) or $BLT1^{-/-}$ were injected s.c. with MRSA for 24 h. Skin biopsies were collected for histological analyses, and 8 μm paraffin-embedded skin sections were used. Cells and skin sections were fixed with 4% formaldehyde for 10 min and permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS) for 10 min. Cells or tissues were then blocked with 1% BSA and 10% normal donkey serum in TBS for 1 h at room temperature and incubated with the indicated primary antibody pairs (ASC versus caspase-1 or ASC versus pBTK [Tyr223]) overnight at 4 °C. Oligonucleotide-conjugated secondary antibodies (PLA probe MINUS and PLA probe PLUS) against each of the primary antibodies were applied, and ligation and amplification were performed to produce rolling circle products. These products were detected with fluorescently labeled oligonucleotides, and the samples were counterstained with Duolink mounting medium with 4′,6-diamidino-2-phenylindole.

Cells were imaged on a Zeiss LSM 510 confocal microscope with an inverted Axiovert 100-M microscope stand using a C-apochromatic 40×/1.2 W correction. Confocal images were taken with identical settings to enable comparison of staining. Z-stacked sections (10–22 slices) of the cells were captured in multitrack, and ImageJ software (NIH, Bethesda, MD) was used to reconstruct the images using the Z project plug-in (29, 30). The extent of association between the inflammasome components and their intracellular localization was quantitated using the JaCoP plug-in for ImageJ (29). The background of the collected images was corrected by the ImageJ rolling ball algorithm plug-in. For each experiment, at least, 100 randomly selected cells or 10 field areas were scored.

Skin Biopsy Dissociation for Flow Cytometry. Skin biopsies were digested with collagenase D (Sigma-Aldrich) and processed to obtain a single-cell suspension, as previously described (25). Cells were stained with the different fluorescent antibodies (as indicated in the figure legends) and acquired on the BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Analyses were performed using FlowJo software (Ashland, OR).

In Vivo Imaging System. In vivo imaging system (IVIS) spectrum/CT (Perkin-Elmer, Waltham, MA) was used to image bioluminescence or fluorescence in the mice skin. Mice were anesthetized with isoflurane and imaged while under anesthesia. Mice were positioned facing the charge-coupled device camera and imaged for the appropriate bioluminescence/fluorescence as follows.

Bioluminescence imaging and analysis: The mice were scanned for up to 4 min to allow for bioluminescent signal detection from each mouse. Mice were scanned longitudinally throughout MRSA skin infection. A region was drawn around each infection, and total flux (photons/s) was measured for each infection site. To obtain a background-free total flux signal, the mouse infection region was subtracted from the background region. The background-free total flux was compared to a standard curve to obtain the bacteria amount in each infection. The standard curve was prepared by spotting known bacterial cfu in TSA plates and injected s.c. in mice and imaged.

DsRed scans and analysis: WT mice that were DsRed served as the autofluorescence background. Mice were scanned, and the Living Image software (Perkin-Elmer) was used to unmix the DsRed signal spectrally. A region was drawn around the spectrally unmixed DsRed signal, and total radiant efficiency was measured ([photons/s]/[μW/cm²]).

Small Interfering RNA Transfection. Immortalized bone marrow-derived macrophages from C57BL/6 mice were transfected using Lipofectamine RNAiMAx (ThermoFisher Scientific) containing 30 nM BTK small interfering RNA (siRNA) or scrambled control (Dharmacon Inc., Lafayette, CO) for 48 h. Macrophages were challenged with 100 ng/mL LPS for 3 h and pretreated with 10 nM LTB_4 for 15 min, followed by inflammasome activation with 1 μM nigericin for 1 h. The culture supernatant was collected, and the cellular messenger RNA (mRNA) isolated to confirm the BTK silencing by qPCR.

In Vivo IL-1β Neutralization. WT mice were pretreated intraperitoneally (i.p.) with 200 μg/mouse of the neutralizing antibodies against IL-1β (B122, Bio X Cell, Lebanon, NH) or isotype control (polyclonal Armenian hamster IgG, hamster polyclonal IgG; BE0091 Bio X Cell) diluted in 500 μL PBS 24 h prior and at the moment of MRSA infection. The skin lesions were scanned for MRSA bioluminescent signal using IVIS Spectrum CT, and the biopsies were collected and analyzed to bacterial load by cfu.

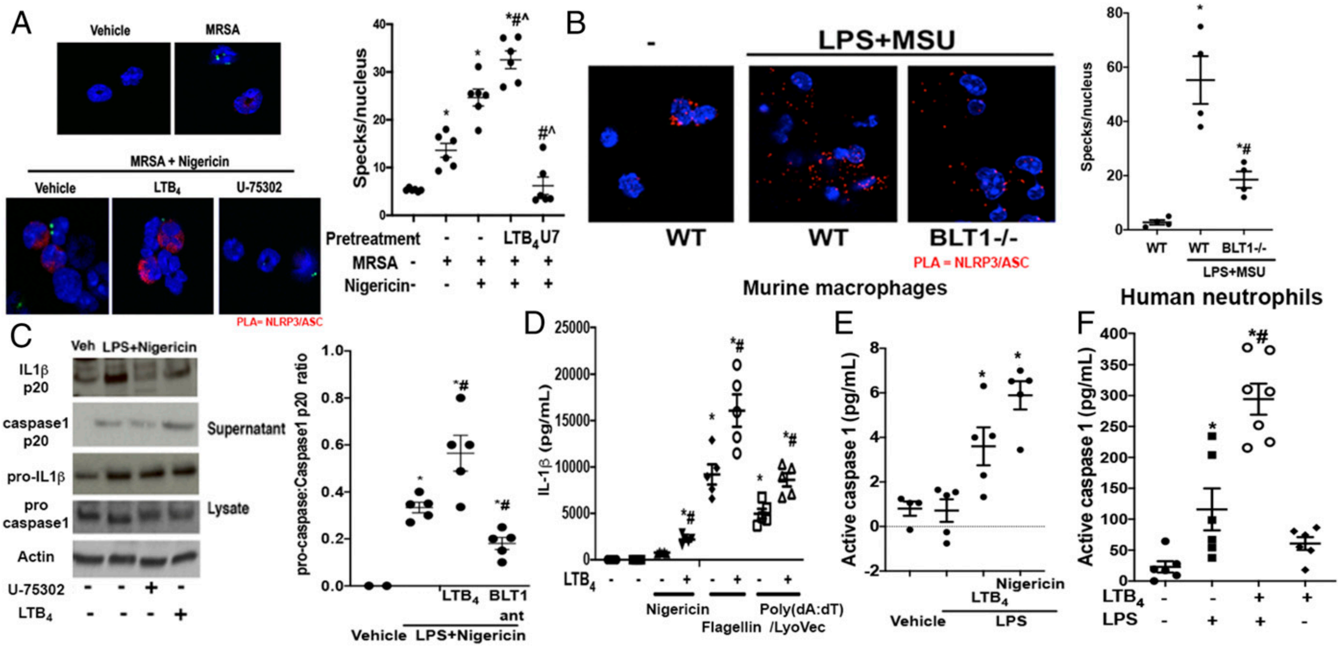

Fig. 2. LTB_4 enhanced inflammasome assembly. PLA was used to detect associations between NLRP3 and ASC represented by the red signal of (*A*, *Left*) Bone marrow neutrophils pretreated with 10 nM LTB_4 for 5 min, 10 μM BLT1 antagonist U-75302 for 30 min, and cultured with or without GFP-MRSA for 3 h followed by 1 μM nigericin for 1 h. *Right*: The average number of specks/nucleus in neutrophils as in *A*. Data are mean ± SEM from, at least, 100 different fields from six mice. $^*P < 0.05$ versus untreated cells. $^\#P < 0.05$ versus MRSA only. $^\wedge P < 0.05$ versus MRSA + nigericin. (*B*, *Left*) Peritoneal macrophages from WT or $BLT1^{-/-}$ mice treated with 100 ng/mL LPS for 3 h followed by 10 μM MSU for 1 h. *Right*, Number of specks/nucleus in macrophages. Data are mean ± SEM from, at least, 10 different fields from four to six mice. $^*P < 0.05$ versus untreated. $^\#P < 0.05$ versus WT-LPS + MSU. In *A* and *B*, each dot represents the average number of specks found in 100 cells harvested from six mice. (*C*, *Left*) IL-1β and caspase-1 cleavage by immunoblotting of macrophages treated with 100 ng/mL LPS and 1 μM nigericin in the presence of LTB_4 and BLT1 antagonist U-75302. Right, Densitometry detection of pro- and active caspase-1. Data are mean ± SEM from at two to five independent experiments mice. Each dot represents the densitometric ratio between pro- and active caspase-1 from two to five mice. $^*P < 0.05$ versus vehicle-treated cells. $^\#P < 0.05$ versus LPS + nigericin-treated cells. (*D*) Macrophages were stimulated with LPS as in *C*, followed by LTB_4 plus different inflammasome activators (1 μM nigericin, 20 μg/mL flagellin, and 2 μg/mL poly [dA:dT]/LyoVec), and IL-1β was determined. Murine macrophages (*E*) and human neutrophils (*F*) were treated with 100 ng/mL LPS for 3 h followed by 10 nM LTB_4 and 1 μM nigericin, and active caspase-1 was measured in the supernatant. (*D*–*F*) Data are the mean ± SEM of five to six individual samples (dots). $^*P < 0.05$ versus untreated cells. $^\#P < 0.05$ versus LPS alone. In all circumstances, quantification of the intensity of the red signal and Western blotting bands was measured by ImageJ analysis, and each dot represents individual experiments or mice.

Statistical Analysis. Data analyses were performed in GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA). The statistical tests used are listed in the figure legends. Briefly, Student's *t* test was used to compare two experimental groups. One-way ANOVA, followed by Tukey multiple comparison correction, was used to compare three or more groups. Two-way ANOVA with repeated measured, followed by Tukey multiple comparison correction, was used to compare infection areas over time between two or more mouse groups. *P* values < 0.05 were considered significant.

Results

LTB_4/BLT1 Axis Promotes IL-1β Production. LTB_4 enhances the production of IL-1β during inflammatory conditions (31). Furthermore, LTB_4 alone or in combination with cytokines and PAMPs activate different transcription factors that further enhance *Il1b* mRNA expression (32, 33). However, whether LTB_4 enhances IL-1β abundance by increasing *Il1b* transcripts or IL-1β processing/maturation via effects on inflammasome activation during infections is not known. Here, we measured *Il1b* transcripts and protein in skin biopsy homogenates from uninfected and MRSA-infected mice. Skin biopsies from WT mice treated topically with LTB_4 or the BLT1 antagonist U-75302 (19) as well as $BLT1^{-/-}$ mice were collected day 1 post-MRSA infection. Our data revealed that topical LTB_4 increased only IL-1β abundance, and BLT1 antagonism inhibited both IL-1β protein and *Il1b* mRNA transcripts during skin infection when compared to WT mice (Fig. 1 *A* and *B*). Furthermore, $BLT1^{-/-}$ macrophages challenged with MRSA produced lower levels of IL-1β when compared to WT macrophages (*SI Appendix*, Fig. S1*A*). These data suggest that topical LTB_4 enhances inflammasome-dependent IL-1β, and endogenously produced LTB_4 during skin infection is required for optimal IL-1β generation. Next, we aimed to determine whether LTB_4 could also amplify IL-1β production in human neutrophils. Here, cells were treated with LPS for 3 h followed by LTB_4 treatment. Our data show that LTB_4 effects also extend to human neutrophils (Fig. 1*E*).

To determine whether endogenously produced LTB_4 influences the kinetics of IL-1β during MRSA skin infection, we employed the IL-1β reporter mouse pIL1DsRED using IVIS technology. The pIL1DsRED mice were infected with MRSA and treated with a topical ointment containing BLT1 antagonist U-75032 or control vehicle (19). DsRed signal was highest at days 1 and 2 postinfection and decreased from day 3 (Fig. 1*C*). This suggests that IL-1β is expressed early during MRSA skin infection. BLT1 antagonist treatment showed minimal expression of DsRed at all points measured during infection, indicating that MRSA-induced LTB_4 production was necessary for IL-1β expression during skin infection. Furthermore, the specific effect of the LTB_4/BLT1 axis in *Il1b* mRNA expression was evidenced by the fact that neither exogenous LTB_4 nor BLT1 antagonist influenced TNF-α secretion in macrophages (*SI Appendix*, Fig. S1*B*) or after infection in the skin biopsy from $BLT1^{-/-}$ mice (Fig. 1*D*).

Next, we aimed to identify in which cells BLT1 is necessary for IL-1β production during MRSA skin infection. pIL1DsRED mice were infected with MRSA and treated with vehicle or BLT1

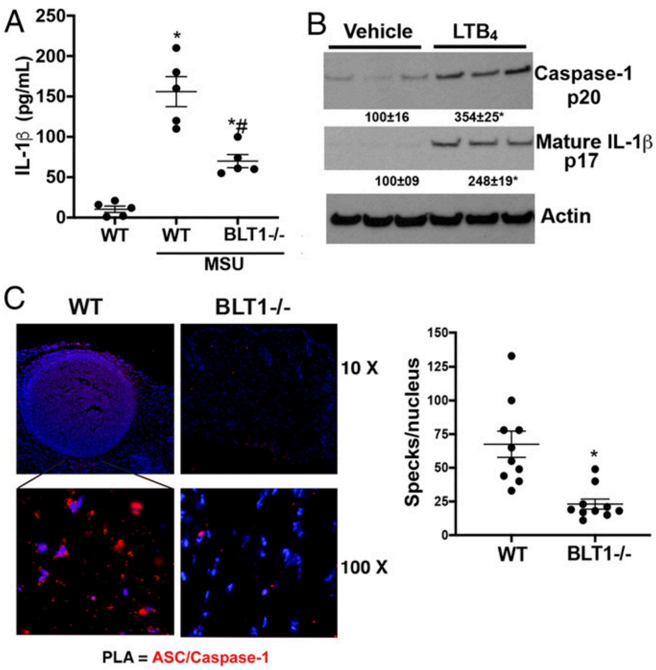

Fig. 3. LTB_4/BLT1 axis is important to inflammasome assembly and IL-1β production. (*A*) Quantification of IL-1β production of WT and BLT1$^{-/-}$ mice inoculated i.p. with 1 mg/mL MSU or PBS. Each dot represents the amount of IL-1β in an individual mouse. Data are the mean ± SEM of five mice. *$P < 0.05$ versus WT + PBS; $^{\#}P < 0.05$ versus WT + MSU. (*B*) Mature IL-1β and activated caspase-1 measured by Western Blot from the skin biopsies of MRSA-infected WT mice treated with an ointment containing vehicle control or LTB_4 (three mice/group) Numbers: Relative mean densitometric analysis of the bands from immunoblots ± SEM (n = five mice/group, in two independent experiments). (*C*, *Left*) In situ PLA of ASC/caspase-1 in the infected skin of WT and BLT1$^{-/-}$ mice. PLA complexes are shown in red, and nuclei are shown in blue. Right, numbers of specks/nucleus in, at least, 10 fields expressed as means ± SEM of 10 mice/group (dots).

antagonist U-75302. BLT1 antagonism did not alter the overall percent of macrophages (F4/80$^+$ cells) in the skin during MRSA skin infection (*SI Appendix*, Fig. S2*A*); however, the percent of macrophages expressing *Il1b* (DsRed$^+$) was significantly lower in the skin of BLT1 antagonist-treated mice than macrophages in the skin of untreated mice (*SI Appendix*, Fig. S2*B*). We observed that both the percent of total F4/80$^+$ macrophages and the mean fluorescent intensity (MFI) of expressing DsRed were reduced with BLT1 antagonist treatment (*SI Appendix*, Fig. S2*C*). BLT1 antagonism decreased the number of global Ly6G$^+$ neutrophils but did not reduce the numbers of DsRed expressing PMNs (*SI Appendix*, Fig. S2 *D* and *E*). Importantly, the MFI of DsRed was lower in mice treated with BLT1 antagonist (*SI Appendix*, Fig. S2*F*), indicating that BLT1 activation was not required for PMN migration to the infected skin but rather necessary for PMN IL-1β expression.

LTB_4 Enhances NLRP3 Inflammasome Activity. We then tested whether LTB_4 controls IL-1β processing through inflammasome assembly and caspase-1 activation. To determine inflammasome assembly, bone marrow-derived neutrophils were imaged by fluorescence microscopy to determine associations of NLRP3 and ASC using PLA, and the close proximity of these proteins resulted in red fluorescence. Untreated neutrophils did not show an association between NLRP3 and ASC (Fig. 2*A*). Neutrophils cocultured with MRSA induced minimal but significant inflammasome assembly. Nigericin-induced inflammasome activation resulted in a high NLRP3/ASC association compared to the vehicle only. While LTB_4 treatment further promoted, the BLT1 antagonist U-75302 treatment inhibited the inflammasome assembly (Fig. 2*A*). To confirm these data, we used another inflammasome activator (MSU) in peritoneal macrophages from WT or BLT1$^{-/-}$ mice; BLT1$^{-/-}$ macrophages treated with LPS plus MSU induced less inflammasome assembly than WT macrophages (Fig. 2*B*).

These findings were confirmed as we observed decreased active caspase-1 and mature IL-1β in the supernatant of macrophages treated with BLT1 antagonist and increased IL-1β in LTB_4 treated cells (Fig. 2*C*). Next, we aimed to investigate whether LTB_4 effects are restricted to NLRP3 or if it also enhances the activation of the AIM2 or NLRC4 inflammasome. Macrophages were challenged with LPS, followed by LTB_4 and the AIM2 activator (Poly [dA:dT]/LyoVec) or the NLRC4 agonist (flagellin). In all circumstances, LTB_4 further increased IL-1β abundance when macrophages were challenged with these other inflammasome activators (Fig. 2*D*). Then, we studied whether LTB_4 itself drives the second signal to activate inflammasome and induces IL-1β secretion. Here, macrophages were treated with LPS as above, followed by LTB_4 for 3 h. We detected more IL-1β levels in the supernatant of macrophages treated with LPS plus LTB_4 when compared to LPS or LTB_4 alone. Furthermore, increased IL-1β in the supernatant correlated with enhanced levels of active caspase-1 (Fig. 2*E*). That LTB_4 itself enhances inflammasome activation was also evidenced in human neutrophils (Fig. 2*F*). These results suggest that LTB_4/BLT1 is necessary for *Il1b* expression and that LTB_4 may be an essential mediator in regulating inflammasome assembly and activation, allowing for mature IL-1β production.

Next, we were poised to test the role of LTB_4 in inflammasome activation in vivo. Initially, we challenged WT and BLT1$^{-/-}$ mice with MSU i.p., and 24 h later, we detected lower IL-1β in the peritoneal lavage of BLT1$^{-/-}$ mice when compared to MSU-challenged WT mice (Fig. 3*A*). We then determined whether LTB_4 production is required for inflammasome activation during MRSA skin infection. WT mice were challenged with MRSA s.c., followed by topical treatment with vehicle control or LTB_4 as we have previously shown (19). Our data show that while MRSA skin infection increases the abundance of both IL-1β and caspase-1, topical LTB_4 further enhances IL-1β secretion and caspase-1 activation in skin lysates (Fig. 3*B*). Importantly, we confirmed the role of BLT1 in inflammasome activation in vivo in skin sections stained for the association between ASC/caspase-1 using PLA. Our data showed decreased ASC/caspase-1 interaction in areas near the abscess in MRSA-infected BLT1$^{-/-}$ mice when compared to infected WT mice (Fig. 3*C*).

Together, these data show that endogenously produced LTB_4 during inflammatory response or skin infection is required for both first and second signals of inflammasome activation.

LTB_4/BLT1 Axis Enhances BTK-Mediated Inflammasome Activation. Inflammasomes are controlled by a variety of posttranslational modifications, including phosphorylation (34). Here, we tested the role of different kinases known to be both activated by LTB_4 and involved in inflammasome activation (34). Macrophages were challenged with LPS, followed by the indicated inhibitors (see Fig. 4 legend), LTB_4, and then nigericin. Our data show that only the BTK inhibitor (ibrutinib) but not PI3K, PKCδ, and Syk inhibitors prevented LTB_4-mediated enhanced IL-1β production (Fig. 4*A*). The role of BTK but not other kinases in LTB_4-mediated inflammasome activation was further confirmed by detecting active caspase-1 in the cell supernatant by immunoblotting (Fig. 4*B*). To confirm the pharmacological findings by gene silencing BTK in macrophages, we employed siRNA as we have previously shown (11–14). Our data show that BTK silencing but not the scrambled control also prevented IL-1β production through LTB_4-enhanced inflammasome activation (Fig. 4*C*).

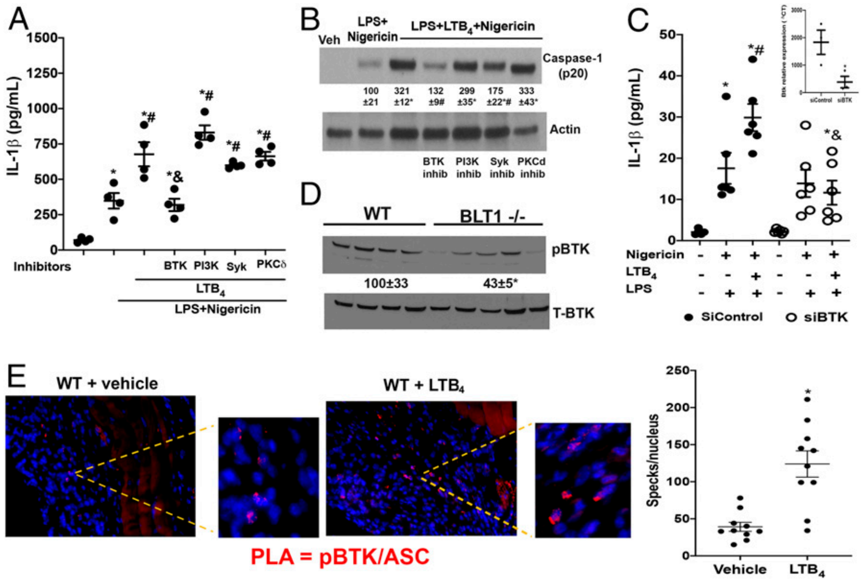

Fig. 4. LTB_4-induced BTK phosphorylation is required for inflammasome assembly. Macrophages were stimulated with 100 ng/mL LPS for 3 h, followed by treatment with the BTK inhibitor (Ibrutinib), PI3K inhibitor (Wortmannin), Syk inhibitor (R406), and PKCδ (Rottlerin) for 20 min, 10 nM LTB_4 for 10 min, and 1 μM nigericin treatment for 1 h. (*A*) IL-1β quantification. (*B*) Western blotting analysis of caspase-1 activation. The numbers represent mean densitometric analysis of the bands shown from three independent experiments. Numbers: mean densitometric analysis of the bands from shown from three independent experiments. (*C*) Immortalized bone marrow-derived macrophages from C57BL/6 mice were treated with BTK siRNA or scrambled control, followed by inflammasome activation and IL-1β detection as in *A*. Data are the mean ± SEM of four to five mice from, at least, two independent experiments. **P* < 0.05 versus untreated cells. #*P* < 0.05 versus LPS + nigericin. &*P* < 0.05 versus LPS + LTB_4 + nigericin. Each dot represents the mean of individual experiments done in triplicate. (*D*) WT and $BLT1^{-/-}$ mice were infected with MRSA for 24 h, and skin lysates were subjected to immunoblotting, followed by detection of total and phosphorylated BTK (Tyr223). Numbers: mean densitometric analysis of the bands from immunoblots ± SEM (*n* = four to five mice/group). (*E*, *Left*) In situ PLA of pBTK (Tyr223) and ASC in the infected skin of WT mice treated topically with vehicle control or LTB_4. PLA complexes are shown in red, and nuclei are shown in blue. Right: numbers of specks/nucleus in, at least, 10 fields expressed as means ± SEM of 10 mice/group. Each dot represents the average number of specks/nucleus in an individual mouse. Data are the mean ± SEM of 10 mice from, at least, two independent experiments. **P* < 0.05 versus mice infected and treated with vehicle control.

Next, to determine whether LTB_4 activates BTK, we assessed BTK phosphorylation and expression during MRSA skin infection. Our data clearly show that BTK is activated during MRSA infection and that LTB_4/BLT1 signaling is required for optimal BTK phosphorylation in the skin (Fig. 4*D*). Given the role of LTB_4 in BTK activation, we aimed to identify whether LTB_4 would enhance BTK and ASC association in vivo. Using PLA to show protein interaction, our data unveiled that MRSA skin infection leads to enhanced phosphorylated BTK and ASC interaction, and importantly, topical LTB_4 treatment further increased ASC/BTK association (Fig. 4*E*). To summarize, we are showing that LTB_4 is required for different steps of inflammasome activation by increasing transcriptional programs as well as phosphorylation of ASC and speck formation.

Crosstalk between LTB_4 and IL-1β to Enhance Skin Host Defense. To demonstrate whether IL-1β is required for LTB_4 effects in microbial clearance, we performed "add-back" experiments by injecting WT and $BLT1^{-/-}$ mice with recombinant IL-1β s.c. at the moment of infection. Our data show that recombinant IL-1β decreases bacterial load in $BLT1^{-/-}$ mice, suggesting that LTB_4 production is not required for IL-1β–dependent microbial clearance in vivo (Fig. 5 *A* and *B*). Next, we determined if IL-1β is necessary for LTB_4-mediated bacterial clearance in the skin. Here, we employed pharmacological and genetic approaches to prevent IL-1β actions in topical LTB_4-treated and infected mice. Our data confirmed previous findings showing that $IL1R^{-/-}$ are more susceptible to MRSA skin infection. Importantly, while topical LTB_4 enhanced microbial clearance in the skin of WT mice treated with the isotype control, LTB_4 did not increase skin host defense in both anti–IL-1β-treated WT or $IL1R^{-/-}$ mice (Fig. 5 *C* and *D*). Together, these data reinforce the role of LTB_4 as a central component for optimal IL-1β production and effects during bacterial skin clearance.

Discussion

Inflammasome activation is a critical component of both host defense and inflammatory responses. Multiple regulatory programs enhance inflammasome activation, including enhancing transcriptional programs, reactive oxygen species production, acetylation, ubiquitination, and phosphorylation (34). However, the signals that trigger upstream kinase activation and amplifies the inflammasome assembly remain to be fully determined. Here, we are moving the field forward by identifying a regulatory program that amplifies both first and second signals required for optimal inflammasome activation and IL-1β production. Our data show that: 1) genetic and pharmacologic modulations of LTB_4/BLT1 axis promote macrophage pro*Il1b* mRNA expression in vivo and in vitro; 2) exogenous LTB_4 enhances inflammasome-dependent IL-1β secretion; 3) both exogenous and endogenous LTB_4 enhance active caspase-1 and mature IL-1β in the supernatant of macrophages and neutrophils from both mice and humans; 4) LTB_4 increases NLRP3 inflammasome assembly in a manner dependent on BTK phosphorylation; 5) LTB_4/BLT1 signaling is required for IL-1β–dependent MRSA skin host defense.

LTB_4 participates in the initiation, potency, maintenance, and endurance of the inflammatory response by regulating different immune cells (35). LTB_4 is a potent inflammatory mediator long known to be a robust neutrophil chemoattractant (36, 37). More recently, new roles for LTB_4 in phagocyte biology have emerged. We and others have shown that LTB_4 is required for phagocytosis and antimicrobial effector functions by increasing reactive oxygen and nitrogen species secretion and defensin production during infection by gram-positive and gram-negative bacteria,

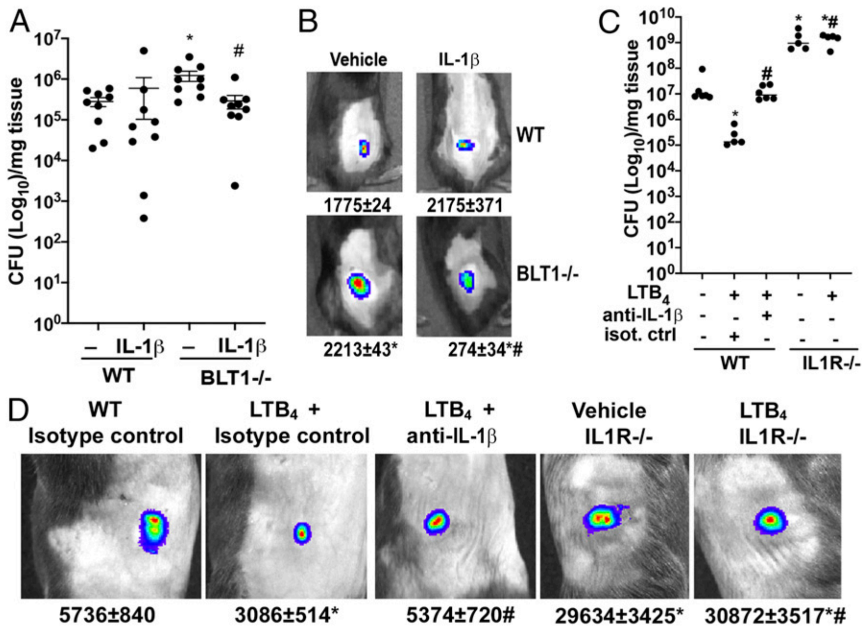

Fig. 5. LTB_4-dependent IL-1β production is required for skin host defense. WT or $BLT1^{-/-}$ mice were injected s.c. with MRSA with or without 50 ng/mL IL-1β followed by determination of cfu (*A*) from the skin biopsies of the animals at 24 h postinfection. (*B*) Representative bioluminescence MRSA quantification in WT and $BLT1^{-/-}$ mice treated or not with IL-1β using the IVIS system. Numbers are relative bioluminescence units in the skin of mice infected as in *A* and imaged at day 1 postinfection. Data are the mean ± SEM of three to four mice/group from three individual experiments. *$P < 0.05$ versus WT. $^{\#}P < 0.05$ versus $BLT1^{-/-}$ treated with vehicle control. (*C*) WT and $IL1R^{-/-}$ mice were treated or not with anti–IL-1β or isotype control 24 h before the infection and at the moment of infection. After infection, mice were treated with topical LTB_4, and the cfu was determined in the skin biopsies. Each dot represents the amount of IL-1β in individual mice in *A* and *C*. (*D*) Representative bioluminescence MRSA quantification in mice infected and treated as in *C*. Numbers are relative bioluminescence units in the skin of mice infected as in *D* imaged at day 1 postinfection. Data are the mean ± SEM of, at least, five mice/group. *$P < 0.05$ versus WT or isotype control. $^{\#}P < 0.05$ versus LTB_4-treated WT mice.

protozoan parasites, and viruses (15–18, 38). Although LTB_4 is an essential homeostatic mediator of the inflammatory response, high and sustained LTB_4 production has been associated with inflammatory diseases and metabolic dysfunctions (35, 39), supporting the idea that abundant and chronic circulating LTB_4 levels are harmful to the host. Moreover, exaggerated LTB_4 is responsible for an aberrant TNF-α production that drives mycobacterial infection susceptibility in a zebrafish model. Also, a gain-of-function single-nucleotide polymorphism in the LTA_4H gene is associated with susceptibility to tuberculosis in humans (40, 41). Furthermore, we have shown that reduced skin infection in diabetic mice is characterized by overwhelming inflammatory response, abundant LTB_4, and IL-1β production and that blockage of BLT1 (but not BLT2) restores IL-1β to basal levels in the infected skin (42). We have also shown that blocking LTB_4 synthesis shows beneficial effects in a model of cecal ligation and puncture-induced sepsis in diabetic mice by decreasing IL-1β and MyD88 expression (28). Here, we are further demonstrating that blocking BLT1 in NLRP3 inflammasome-activated macrophage from diabetic mice inhibited caspase-1 activation (*SI Appendix*, Fig. S3 *A* and *B*).

Although we and others have shown that IL-1β participates in detrimental LTB_4 responses, whether LTB_4 differently regulates the first and/or the second signals required for inflammasome-dependent IL-1β maturation is unknown. LTB_4-induced IL-1β production and NLRP3 inflammasome activation has been shown during *Leishmania amazonensis* infection (43) and in a model of arthritis (20, 21). However, to date, no mechanistic studies have been performed to address this critical question.

LTB_4 controls the expression and actions of different PRRs, oxidant generation, protease release, and activation of inflammatory transcription factors (12, 13, 30, 32, 33, 44). Moreover, LTB_4 increases NF-κB activation that could, in turn, enhance IL-1β, NLRP3, and caspase-1 expression. Here, using a reporter mouse that detects *Il1b* mRNA expression (pIL1bDsRed) (3, 12), we show that BLT1 antagonism inhibits *Il1b* transcription in both macrophages and neutrophils at the peak of expression postinfection. This effect was restricted to IL-1β since we did not witness an effect of topical LTB_4 or BLT1 antagonist on TNF-α production in macrophages or during MRSA skin infection. Since NF-κB controls the expression of both IL-1β and TNF-α, the specific checkpoints underlying the effects of LTB_4 in *Il1b* expression remain to be determined.

We and others have shown that the LTB_4/BLT1 axis either directly induces or further amplifies the activation of different kinases, shaping cellular responses in different environments (44–46). The inflammasome is activated by signals derived from pathogen products and host-derived molecules (DAMPs). Therefore, LTB_4 expands the list of endogenous molecules that activate the inflammasome. However, our paper stems from the fact that LTB_4 is not secreted by dying cells. LTB_4 is quickly produced (seconds to minutes) after cell activation and, therefore, could further amplify the signals required for inflammasome assembly. Here, we showed that LTB_4 amplifies the effects of different NLRP3 agonists as well as induces inflammasome assembly directly.

Given the role of BLT1 in activating a myriad of kinases, including those known to enhance inflammasome activation (47–49), we tested whether BLT1 participates in the NLRP3 inflammasome assembly. Our data show that BLT1 activation is required for both in vivo and in vitro inflammasome activations using a variety of systems and activators, such as MSU, nigericin, and MRSA-induced inflammasome activation, which shows the broad effects of LTB_4 in inflammasome activation. The role of eicosanoids in inflammasome activation remains inconclusive. While prostaglandin E_2 enhances adenosine 3′,5′-cyclic monophosphate/protein kinase A (cAMP/PKA)-dependent NLRP3 phosphorylation in macrophages, PKA inhibits NLRP3 activation in human monocytes and macrophages (3, 50).

BLT1 is coupled to $G_{\alpha i}$ and $G_{\alpha q}$ inhibiting cAMP and increases Ca^{2+} mobilization, respectively (14, 51, 52). Importantly, GPCRs that are coupled to either $G_{\alpha i}$ or $G_{\alpha q}$ are also known to activate the inflammasome by directly modulating the abundance of second messengers or by influencing kinase-mediated inflammasome association (53). Although we did not investigate the role of different G proteins in BLT1-mediated inflammasome activation and the role of downstream effectors, the fact that neither PI3K nor PKC-δ inhibition prevented LTB_4-mediated inflammasome activation suggests that $G_{\alpha i}$-dependent decreased cAMP might be allowing inflammasome assembly. Whether cAMP influences BTK activation remains to be determined.

Here, we are providing evidence that LTB_4/BLT1/BTK phosphorylation is required for NLRP3/ASC assembly in phagocytes. Our data show that LTB_4-mediated BTK1 phosphorylation is needed for the NLRP3/ASC association during inflammasome activation and MRSA skin infection. BTK is a tyrosine kinase necessary for B cell receptor and Fc receptor signaling (54). More recently, the role of BTK in phagocytes has emerged. BTK activation is required for toll-like receptors signaling, phagocyte recruitment to the inflammatory site, and macrophage maturation (55, 56). BTK is phosphorylated in two tyrosine sites (Y223 and Y551), and Syk and Lyn are known to be upstream to BTK in B cells (57). Whether these kinases are involved in BTK actions in macrophages is unknown. Here, we showed that Syk inhibitor R406 did not prevent LTB_4-mediated inflammasome, which suggests that Syk does not influence BTK actions in inflammasome actions. BTK classically activates PLC, PI3K/Akt, and NF-κB, which could, in turn, regulate the first signal (NF-κB–dependent expression of inflammasome components) as well as the second signal (PI3K) and, therefore, increasing NLRP3 phosphorylation and favoring inflammasome assembly. However, PI3K inhibition does not prevent LTB_4 effects on inflammasome assembly. These data suggest that LTB_4 enhances inflammasome assembly by increasing the association between phosphorylated BTK with ASC. Future studies will be needed to determine the events upstream to BTK activation as well as the ASC phosphorylation sites activated by BTK.

IL-1β is crucial for controlling *S. aureus* skin infection by driving neutrophil migration to the site of infection and abscess formation (58). We have demonstrated that LTB_4 enhances *S. aureus* clearance in the skin by increasing neutrophil migration and abscess formation (19). Importantly, it has been shown that LTB_4 is produced as part of the inflammasome activation, an event called the "eicosanoid storm" (59). Because of the potential crosstalk between LTB_4 and IL-1β during the inflammatory response, we determined if there was a codependency between these two molecules during skin infection. Here, we provide evidence that LTB_4 enhances IL-1β production to improve host defense and that IL-1β increases microbial clearance independent of LTB_4 production. These findings are exciting and somewhat expected since LTB_4 is produced within minutes to hours after stimuli, while IL-1β is dependent on gene transcription and inflammasome action. Therefore, our findings show a two-wave step required for skin host defense mediated by an early production of LTB_4 that further amplifies IL-1β–dependent neutrophil migration, abscess formation, and wound healing during *S. aureus* infection.

To summarize, our data show that the prevention of exaggerated LTB_4/BLT1 actions is a promising and potent therapeutic strategy to prevent overwhelming inflammatory response by inhibiting both transcriptional programs involved in the expression of inflammasome components as well as preventing the intracellular signals required for inflammasome assembly. On the other hand, the topical treatment with exogenous LTB_4 is a promising candidate to increase both local antimicrobial effector functions and inflammatory response in hard to treat infections.

Data Availability. All study data are included in the article and *SI Appendix*.

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