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Nonredundant Roles for Leukotriene B4 Receptors BLT1 and BLT2 in Inflammatory Arthritis

Steven P. Mathis,*† Venkatakishna R. Jala,*† David M. Lee,‡ and Bodduluri Haribabu*†

Lipid mediators derived from arachidonic acid through the cyclooxygenase and lipoxygenase pathways are known to be important mediators of inflammation. Studies in mouse models demonstrated an important role for the high-affinity leukotriene B4 receptor BLT1 in arthritis, atherosclerosis, and asthma. BLT2, a low-affinity leukotriene B4 receptor, was also shown to be a high-affinity receptor for cyclooxygenase-1 derived 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid. However, its biochemical activities and physiological roles remain unknown. In this study, we developed mice deficient in BLT2 by targeted disruption. The BLT2−/− mice developed normally, and analysis of immune cells showed that disruption of BLT2 did not alter BLT1 expression or function. Mast cells from the C57BL/6 mice but not from the BLT2−/− mice showed intracellular calcium mobilization in response to 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid. In an antibody-induced inflammatory arthritis model, the BLT2−/− mice showed reduced incidence and severity of disease, including protection from bone and cartilage loss. Reciprocal bone marrow transplant experiments identified that loss of BLT2 expression on a bone marrow-derived cell lineage offers protection against severe disease. Thus, BLT2, a unique receptor for 5-lipoxygenase- and cyclooxygenase-1-derived lipid mediators, represents a novel target for therapies directed at treating inflammation associated with arthritis.


L ukotriene B4 (LTB4) is a potent lipid mediator of inflammation produced through the sequential actions of 5-lipoxygenase, 5-lipoxygenase activating protein, and leukotriene A4 hydrolase (1). LTB4 mediates diverse biological activities through two distinct highly conserved G protein-coupled receptors BLT1 and BLT2 (2, 3). BLT1, the high-affinity receptor, is expressed on neutrophils, eosinophils, monocytes, dendritic cells, and activated T cells to control migration of these cells to sites of inflammation (4–8). Development of BLT1−/− mice allowed delineation of its role in the progression of many inflammatory disorders, including arthritis, atherosclerosis, asthma, and host response to infection (5, 7, 9–12). In contrast, very little is known about the biological responses mediated via BLT2.

In humans, BLT2 is widely expressed in a variety of immune cell types, including neutrophils, monocytes, and mast cell-derived immortalized hMC-1 cells (13). Although functional expression was difficult to determine in primary cells because of the presence of the high-affinity receptor BLT1, studies in cell lines clearly showed that the BLT2/LTB4 axis mediated intracellular calcium mobilization and chemotaxis (3, 11). HUVEC cells were also shown to express functional BLT2 when treated with TNF-α (14). Human BLT2 was recently implicated in IL-1β–stimulated IL-8 synthesis in mast cells (15). However, the expression and function of murine BLT2 remains unclear. Although initial studies implicated BLT2 expression in murine keratinocytes and small intestine, recent studies suggest that BLT2 is expressed in mast cells (13, 16). A novel cyclooxygenase-1 derived ligand 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid (12-HHT) was shown to be the high-affinity ligand for BLT2 (16).

To define the biological activities of BLT2 and to explore the relevance of this receptor to inflammatory diseases known to be mediated by LTB4, we generated a BLT2-deficient mouse line. Because the BLT1 promoter is located within the coding region of BLT2, we chose to disrupt this gene by insertion rather than deletion. In the current study, we show that BLT2 knockout mice express normal levels of BLT1 but are protected from disease development in the K/BxN model of inflammatory arthritis. Using bone marrow-reconstitution experiments, we show that expression of BLT2 is necessary on bone marrow-derived cells for full arthritis development. Furthermore, experiments in transfected cell lines and human synovial neutrophils demonstrate that BLT2 may use signaling by LTB4 and 12-HHT in vitro. However, LTB4 seems to be the preferred ligand for inducing migration through BLT2.

Materials and Methods

Mice

The BLT1−/− mice on the BALB/c and C57BL/6 backgrounds and BLT1/BLT2 double-deficient mice were described earlier (7, 8, 11). The BLT2−/− mice were generated at the Duke University Transgenic Mouse Core Facility (Durham, NC) and were subsequently backcrossed onto the C57BL/6 background for seven generations. All studies and procedures were approved by the Animal Care and Use Committee of University of Louisville Research Resources Center.

Targeting construct and generation of BLT2-deficient mice

The previously described BLT1/BLT2-targeting construct (11) was modified to target only the BLT2 gene. The short arm in the BLT1/BLT2 double-knockout construct was replaced with a fragment from the BLT2-coding region starting at 31 bp. The resulting final construct pWSGB2 was 16 kb in length. AK7 (129S4/SvJaeSor) ES cells (107) were electroporated with 25 μg Not-I linearized WSGB2-DNA. The transfected cells were grown in DMEM media with 200 μg/ml G418 and 2 × 10−6 M ganciclovir for 10 d. Surviving clones were tested for recombination using a neomycin-coding

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sequence primer (TGGCAGGCGATGCGCTCCCTTAGCTG). Of the several positive clones, four were expanded, and the genotypes were confirmed by Southern blot analysis with 10–20 μg DNA using a BamHI-EcoRI fragment external to the 3′ end of the knockout construct as a probe. Two undifferentiated clones were individually micro-injected into C57BL/6 blastocysts and transferred into pseudo-pregnant C57BL/6 mice. Chimeric mice generated from one of these clones resulted in immediate germline transmission, and the F-1 (C57BL/6 and 129 SvJ) offspring were used to establish the mouse colonies. Genotyping was performed by Southern blotting or more routinely using a three-primer PCR reaction with the primers 5′-ATGGCTGACTATGCTTTACC-3′, 5′-AGTTGCAGACCAAGTGTGGC-3′, and 5′-CAGCTGACCCAGAGCATGG-3′. All mice were housed in a specific pathogen-free barrier facility.

RNA isolation, reverse transcription, and PCR

Total RNA was isolated from bone marrow cells using TRIzol reagent, followed by a mini RNeasy kit from Qiagen (Valencia, CA). Total RNA was treated with Turbo DNAase from Ambion (Austin, TX) to remove the traces of genomic DNA contamination from RNA samples. cDNA was synthesized from 250 ng total RNA with random hexamer primers using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA). The mBLT1, mBLT2, and β-2 microglobulin (control gene) genes were PCR amplified by HotStart Taq Polymerase (United States Biochemical [Cleveland, OH]) using the above-synthesized cDNA (2 μl/reaction) as a template, with the following primers, in 20 μl total reaction volume. PCR was also performed on the cDNA reaction mix that did not contain reverse transcriptase as a negative control (data not shown). murine BLT1: forward primer: 5′-ATGGCTGACCAACATCATCTTCC-3′, reverse primer: 5′-CCTCGATCCCAGTAG-3′; murine BLT2: forward primer: 5′-ATGGCTGACTATGCTTTACC-3′, reverse primer: 5′-AGTTGCAGACCAAGTGTGGC-3′, and murine β-2 microglobulin: forward primer: 5′-CATGCGGCTCAGATGGTACAC-3′, reverse primer: 5′-GATCCATGT-TCTCGACCCAGTAG-3′. The PCR conditions used to amplify these products were as follows. The cDNA (2 μl) and forward and reverse primers (7.5 pmol) were added to PCR tubes containing 0.2 mM deoxynucleoside 5′-triphosphate, 1.5 mM MgCl2, and 0.2 U HotStart Taq DNA polymerase along with the PCR buffer provided with the enzyme at 1× concentration. The PCR conditions used were template denaturation at 95°C for 4 min, followed by 45 cycles at 95°C for 30 s (denaturation), 56°C (annealing), and 72°C for 30 s (extension). The reaction was continued for 5 min to complete the extension. The expected sizes of the products for murine BLT1, murine BLT2, and β-2 microglobulin were 319, 191, and 73 bp respectively.

Calcium flux

Calcium mobilization was monitored in Indo-1-loaded cells stimulated with LTB4 at the indicated concentrations. Each experiment represents an analysis of 4 × 106 cells. Murine neutrophils were elicited with zymosan for 4 h and were flushed from the peritoneum in wash buffer. Bone marrow-derived mast cells were generated as described previously (17). Briefly, bone marrow was flushed from femurs and tibia of donor mice and cultured in the presence of 10 ng/ml recombinant murine IL-3 (Pierce, Rockford, IL) and 12.5 ng/ml recombinant murine stem cell factor-1 (R&D Systems, Minneapolis, MN) for 4 wk. Cells were then spun down, resuspended at 2 × 106/ml in 1× PBS + 1% BSA, loaded with 1.5 μl pluronic acid (200 mg/ml in Me2SO) and 2 μl Indo-1 (1 μM solution), and incubated for 30 min at 37°C. After incubation, the cells were washed with 1× PBS + 0.1% BSA. The cells (2 × 106 cells/ml) were resuspended in HBSS containing 1 μM CaCl2. Calcium traces were recorded in a HITACHI fluorescence spectrometer (model-F-2500) with an excitation wavelength of 355 nm and an emission wavelength of 405 nm.

Chemotaxis

Migration of 300,19 cells was evaluated using 5-μm pore size Transwell filters (Costar, Cambridge, MA). The lower chamber was loaded with LTB4 or 12-HHT at various concentrations in RPMI 1640 medium with 1% FBS in a volume of 600 μl. The 300,19 cells expressing either BLT1 or BLT2 (1 × 106 cells in 100 μl RPMI 1640, 1% FBS) were placed into the upper chamber. After 3 h of incubation at 37°C in 5% CO2, the upper chamber was removed, and cells, which migrated into the lower chamber, were counted in a Bürker chamber.

Human synovial fluid leukocyte analysis

Human knee synovial fluids were obtained as discarded material from patients with active rheumatoid arthritis (RA) undergoing diagnostic or therapeutic arthrocentesis. Arthritis diagnosis was ascertained by an American Board of Internal Medicine-certified rheumatologist and/or by review of laboratory, radiologic, and clinic notes and by applying American College of Rheumatology (Atlanta, GA) classification criteria (18). All studies received Institutional Review Board approval. The synovial leukocytes (>95% neutrophils) were washed with RPMI 1640 for chemotaxis experiments or with PBS + 0.1% BSA for calcium flux experiments. The cells were treated as above for calcium flux experiments; the addition of CP-105,096 or L-255,819 was the only difference. Chemotaxis with these cells was performed as above, except that 5 × 105 cells were used per well, and the chemotaxis buffer contained 0.5% FBS.

Flow cytometry

Blood was taken and washed in 1× PBS + 0.1% BSA (wash buffer). The cells were then incubated with anti-mouse CD16/32 (BD Pharmingen, San Diego, CA) for 30 min at 4°C. The cells were washed and incubated for 45 min at 4°C with biotinylated mouse anti-mouse BLT1 (3D7). The cells were washed and incubated with CD3-FITC, B220-PE, NK1.1-PE, Siglec-F-PE, and CD11b-FITC (BD Pharmingen). Streptavidin-allophycocyanin (BD Pharmingen) was also added for 3D7 staining. The cells were incubated for 45 min at 4°C and washed. BD FACs Lyse Solution was then added for RBC lysis and fixation for 1 h. The cells were washed, resuspended for analysis on a FACS Calibur Cytometer (BD Biosciences, San Jose, CA), and analyzed with FlowJo software.

K/BxN arthritis

Serum from K/BxN arthritic mice (19) was pooled to assure uniformity of the injections in each group of an experiment. One hundred microliters of serum was injected i.p. on days 0 and 2, and mice were evaluated for clinical symptoms starting at day 0. The paw and ankle measurements were taken using a pocket thickness gauge (Mitutoyo, Aurora, IL). All mice were examined at least every 2 d following the appearance of disease. Arthritis of each individual limb was graded using the following scoring system: 0, normal; 1, apparent swelling and redness limited to one digit; 2, swelling in more than one digit; 3, severe redness and swelling of the entire paw; and 4, maximally inflamed limb with involvement of paw and ankle joint. The maximum score per mouse ranged from 16 (20). Mice were scored as arthritic if the clinical score was ≥2 on consecutive days.

Bone marrow transfer

Recipient mice were irradiated with 900 rad. The following day, donor mice bone marrow was flushed from the femur and tibia with RPMI 1640 and washed with 1× PBS and resuspended in 1× PBS. The bone marrow from one donor mouse was reconstituted with one recipient mouse. Mice were housed in sterile cages for ≥4 wk. Bone marrow was allowed 8 wk to engraft before performing arthritis experiments.

Histopathology

Upon termination of the experiment, mice were sacrificed, and joint tissues were placed in buffered 10% formalin for 16 h, followed by decalcification, dehydration, and embedding in paraffin. Joint sections (5 μm) were stained with H&E and toluidine blue for histologic examination. Arthritis severity was scored, as described previously, with each parameter having a maximum score of 5 (20). Representative pictures were taken using a Nikon Eclipse E400 microscope.

Statistics

Experimental groups contained at least five mice. Experiments involving mice were repeated at least three times. Statistical significance was determined using the Mann-Whitney U test.

Results

Generation and characterization of BLT2−/− mice

The BLT1−/− and BLT1+/−/BLT2−/− mice were described previously (7, 11). The genomic locus spanning the BLT1 and BLT2 genes and the strategy for making the BLT2−/− mice are shown in Fig. 1A. Genomic Southern blots with an EcoRI and BamHI fragment 3′ to the targeting construct showed the expected fragment sizes for BLT2−/− and wild-type (WT) (Fig. 1B) mice. Three primer PCR reactions were developed for determining the genotypes at the BLT1 and BLT2 loci. Fig. 1C shows the genotyping of all three BLT-deficient mice (BLT1−/−, BLT1−/−/BLT2−/−, and BLT2−/−).
whereas mast cells from both mice responded to LTB4 (Fig. 1). Mobilization of intracellular calcium in response to 12-HHT (Fig. 1) is shown in Fig. 2. A phagocytes, NK cells, B cells, and T cells in peripheral blood are the expression of BLT1 on neutrophils, eosinophils, macrophages, and BLT2 was tested in the total RNA isolated from the bone marrow of the WT and the indicated BLT1 receptor knockout mice. Calcium flux was monitored in Indo-1-stained, bone marrow-derived mast cells (4 × 10^6) from WT or BLT2^−/− mice stimulated with 100 nM 12-HHT (E) or 100 nM LTB4 (F). EGFP, enhanced GFP.

Real-time PCR analysis showed that total RNA from bone marrow of WT mice was positive for BLT1 and BLT2, and each of the knockout mice lacked the expression of corresponding RNA (Fig. 1D). Recent studies demonstrated that murine BLT2 is expressed in mast cells, and 12-HHT is a high-affinity ligand for BLT2 (16). Bone marrow-derived mast cells from WT mice, but not from BLT2^−/− mice, mobilized intracellular calcium in response to 12-HHT (Fig. 1E), whereas mast cells from both mice responded to LTB4 (Fig. 1F).

Because the BLT1 promoter is within the coding region of the BLT2 gene, we sought to determine the consequence of BLT2 disruption on the expression of BLT1. We analyzed the expression patterns of BLT1 using a murine BLT1-specific mAb in the WT and BLT2^−/− mice, while using BLT1^−/− mice as a negative control. The expression of BLT1 on neutrophils, eosinophils, macrophages, NK cells, B cells, and T cells in peripheral blood are shown in Fig. 2A. BLT1^−/− (dotted line) cells showed no expression of BLT1 in any cell type, as would be expected. WT (solid line) and BLT2^−/− (dashed line) mice showed similar BLT1 expression levels in neutrophils, eosinophils, and macrophages, and they both lacked measurable expression in NK cells, B cells, and T cells. There was no significant difference in the total numbers of each cell type seen in the blood. Similar patterns of expression were seen in cells collected from bone marrow, spleen, and lymph nodes (data not shown).

Chemotaxis and calcium-mobilization assays of zymosan-elicited neutrophils showed that WT and BLT2^−/− neutrophils displayed similar LTB4 dose-response profiles in both assays (Fig. 2B, 2C). Neutrophils from BLT1^−/− and BLT1/2^−/− mice lacked chemotaxis (data not shown) or calcium response to LTB4 at all concentrations tested (Fig. 2C).

**BLT2^−/− mice demonstrate attenuated inflammatory responses in autoantibody-driven arthritis**

To determine the consequence of the loss of BLT2 in an inflammatory disease, we tested these mice in the K/BxN serum-transfer model of inflammatory arthritis (21). In this model, BLT2^−/− mice developed an attenuated disease, reaching only approximately half the clinical score of WT mice. As control, we also quantified arthritis responses in BLT1^−/− mice, which, as previously demonstrated in an independent BLT1^−/− strain, showed only transient signs of disease (9) (Fig. 3A). Measurements of the paw (Fig. 3B) and ankle (Fig. 3C) thickness showed a similar trend as the clinical score, with BLT2^−/− mice developing partial disease. Histopathological scoring of H&E-stained joint sections showed a large influx of inflammatory cells and bone erosion in WT mice, whereas inflammation and bone erosion were absent in the joints of BLT1^−/− mice (Fig. 3D–F). BLT2^−/− sections showed reduced influx of inflammatory cells and absence of bone erosion (Fig. 3D–F). Toluidine-stained joint sections showed widespread loss of synovial cartilage staining and thickness only in the WT mice but not in BLT1^−/− or BLT2^−/− mice (Fig. 3D, 3G).
**FIGURE 2.** Functional expression of BLT1 in BLT2 knockout mice. 3D7, a mouse-specific BLT1 mAb, was used to stain peripheral blood from WT (solid line), BLT1−/− (dotted line), and BLT2−/− (dashed line) mice along with cell lineage markers. A, Staining for neutrophils, eosinophils, macrophages, NK cells, B cells, and T cells. B, Transwell chemotaxis of zymosan-elicited neutrophils from WT and BLT2−/− mice. Neutrophils were placed in the upper chamber and allowed to migrate through a 5-μm filter toward varying concentrations of LTB4. Calcium flux was monitored in Indo-1–loaded, zymosan-elicited peritoneal neutrophils (4×10^6) stimulated with various concentrations of LTB4.

**BLT2 expression on cells of hematopoietic origin is required for the development of arthritis**

To examine the cell-type specificity of BLT2 expression for arthritis development, bone marrow-transplantation studies were undertaken. WT mice congenic for CD45.1 were used to enable the determination of proper engraftment of bone marrow. After 8 wk of reconstitution, analysis of CD45.1 and CD45.2 (C57BL/6) expression on peripheral blood leukocytes showed ≥94% of the cells were of donor origin (Supplemental Fig. 1). Eight weeks following bone marrow reconstitution, mice were injected with arthritogenic serum. When engrafted with the WT bone marrow, WT or BLT2−/− mice showed disease patterns similar to naive WT mice, whereas WT mice engrafted with BLT2−/− bone marrow were protected from disease development (Fig. 4A, 4B). WT mice engrafted with BLT2−/− bone marrow also demonstrated a reduction in paw (Fig. 4C) and ankle (Fig. 4D) swelling. Histopathological scoring of bone marrow transfer-recipient mice showed a significant decrease in inflammatory cells in WT mice given BLT2−/− bone marrow (Fig. 4E), whereas BLT2−/− mice receiving WT bone marrow displayed similar levels of inflammation as naive WT mice. Mice receiving the BLT2−/− bone marrow were also protected from bone erosion (Fig. 4F) and cartilage damage (Fig. 4G).

**Activation of BLT2 by 12-HHT and LTB4**

To examine the differential activation of BLT1 and BLT2 by 12-HHT and LTB4, we generated 300.19 cells expressing BLT1 or BLT2. LTB4 or 12-HHT was used as the chemoattractant for 300.19-BLT1 and BLT2 cells in Transwell chemotaxis. As was shown previously, LTB4, but not 12-HHT, stimulates chemotaxis of cells expressing BLT1. Although 12-HHT displayed a higher affinity than LTB4 for BLT2, it is a very weak agonist in chemotaxis assays, with <3% activity relative to LTB4 (Fig. 5A, 5B). Calcium mobilization with these same cells showed that 12-HHT displayed the same efficacy at inducing intracellular calcium flux as LTB4 in cells expressing BLT2 (Fig. 5C).

To explore the potential interplay of BLT1 and BLT2 in human RA, we examined the effects of LTB4 receptor inhibitors on leukocytes from human RA synovial fluids where neutrophils constitute >95% of these cells. Previous studies showed that CP-105,696 is a specific inhibitor of BLT1 (22). LY-255,283 is a BLT2-specific inhibitor at low concentrations, but it inhibits BLT1 and BLT2 at a concentration >1 μM (22). Human RA synovial fluid neutrophils were tested in Transwell chemotaxis assays using LTB4 as the chemoattractant with increasing concentrations of CP-105,696 or LY-255,283. Neutrophils demonstrate a wide peak in response to LTB4. There is a right shift of the chemotaxis peak response with increasing concentrations of CP-105,696, and complete inhibition of migration is seen only at high doses of inhibitor (10 μM) (Fig. 6A). Similar experiments using LY-255,283, a BLT2-specific inhibitor, demonstrated loss of chemotaxis only when the inhibitor concentration reached 10 μM (high dose), at which concentration it is also known to inhibit BLT1 (Fig. 6B).

LTB4-induced calcium-mobilization response was analyzed to determine the differential sensitivity of synovial neutrophils to BLT1 and BLT2 inhibitors. At low concentrations of LTB4 (3 nM), the CP-105,696 compound was highly effective in blocking calcium signaling (Fig. 7A). However, much higher inhibitor levels are required to block calcium release at concentrations...
expected in the activated RA synovium (Fig. 7B). In contrast, the LY-255,283 compound only inhibits calcium mobilization of synovial neutrophils when it reaches a concentration that inhibits BLT1 as well as BLT2 (Fig. 7C,7D).

Discussion

The deletion of the BLT2 gene reported in this study reveals a potential role for this receptor in inflammatory arthritis. Previous studies outlined an important role for LTB4 and its high-affinity receptor BLT1 in distinct models of arthritis (9, 11, 23). The current studies using a newly developed BLT2−/− mouse line demonstrates reduced incidence and severity of inflammatory arthritis in the presence of a fully functional 5-lipoxygenase pathway and the high-affinity LTB4 receptor BLT1. Thus, BLT2 plays a nonredundant role in the development of an inflammatory disease.

The BLT2−/− mice were viable, developed normally, and displayed no overt behavioral or morphological defects. The Southern blot and PCR analysis of mice and the demonstration that bone marrow-derived mast cells from these mice lacked the expression of a functional 12-HHT receptor clearly establishes the generation of BLT2−/− mice. Although macrophages and, to some extent, neutrophils were suggested to express BLT2 (3, 24), and BLT2 mRNA was detected in a wide variety of human tissues, the expression of BLT2 in mouse tissues is unclear. The Luster laboratory failed to detect murine BLT2 mRNA by Northern blotting in neutrophils, macrophages, T cells, lymph node, spleen, or lung (25), and our laboratory could not detect mBLT2 in neutrophils, macrophages, spleen, or liver (11). The relative abundance of human and murine leukotriene receptor cDNA clones in public-expressed sequence tag databases suggests that although human BLT1 and BLT2 are expressed at similar abundance (43 and 41 clones, respectively), murine BLT1 is more frequently identified than murine BLT2 (22 versus 3 clones). These observations suggest that although the mBLT2 gene is indeed expressed, its level of transcription is very weak relative to human BLT2. The recent discovery that 12-HHT is an exclusive ligand for BLT2 allowed us to demonstrate functional expression of BLT2 in mast cells and loss of this 12-HHT–mediated calcium mobilizing receptor in the BLT2−/− mice, while retaining a similar response to LTB4.

A knockout of BLT2 has the potential to disrupt the expression of BLT1 because the promoter of BLT1 is located within the coding region of BLT2. The coding region of BLT2 was disrupted but left intact to avoid such a problem. A murine BLT1 mAb (3D7) allowed us to demonstrate that BLT1 expression was similar in WT and BLT2−/− mice. Abundant expression of BLT1 was detected in neutrophils, eosinophils, and macrophages from WT and BLT2−/−
mice. As might be expected, resting T cells and B lymphocytes did not show detectable expression of BLT1. However, using 3D7, we previously demonstrated expression and regulation of BLT1 in CD8+ T effector cells (26). Expression was similar to WT mice, but the function of BLT1, as seen in chemotaxis and intracellular calcium-mobilization assays, in the BLT2−/− mice was also similar to WT mice.

A large body of data suggests a role for LTB4 in human RA (27–30), and several studies in diverse mouse models also suggested an important role for leukotriene biosynthetic pathways and LTB4 receptors in the development of inflammatory arthritis (9, 11, 23, 31, 32). However, the clinical failure of drugs targeting this pathway in RA raised doubts regarding the functional importance of LTB4 in disease (33). Patient data demonstrate that the level of LTB4 in the joint positively correlates with disease severity, and mRNA levels for its two receptors are increased (29, 30). BLT1 expression (9). The observation that neutrophil recruitment remains robust in the absence of BLT1 in established arthritis pointed to a possible role for BLT2 when LTB4 levels are higher in the joint. A knockout of BLT2 enables us to determine whether the receptor plays a role in the arthritis disease process.

The K/BxN model of arthritis has proven to be useful in determining which cells and mediators are important in the effector phase of arthritis, by bypassing the steps of Ag processing and display of autoreactive peptides on dendritic cells and Tand B cell involvement. In previous experiments, because BLT1−/− and BLT1/BLT2−/− mice were protected from the development of collagen-induced arthritis, it was not clear whether BLT2 played a role in arthritis (11). The results presented in this article clearly show that BLT2−/− mice were protected from disease development in the K/BxN arthritis model. This protection was visualized in sectioning of the ankle joint, where a large influx of inflammatory cells and bone and cartilage destruction were seen in joints of WT mice, whereas little inflammation and no bone destruction were seen in BLT2−/− sections. Our results also confirm the data of Kim et al. (9), who showed protection conferred by the loss of BLT1. Thus, there is an apparent nonredundant requirement for BLT1 and BLT2 in autoantibody-induced arthritis.
Because the expression of BLT2 in mice is not clearly established, it was difficult to infer the cell-type specificity of BLT2 expression required for the induction of arthritis. Bone marrow-transfer experiments clearly demonstrated that WT and BLT2<sup>−/−</sup> mice receiving WT bone marrow developed arthritis at similar levels. WT mice receiving BLT2<sup>−/−</sup> bone marrow were protected from development, similar to BLT2<sup>−/−</sup> mice. Histopathological examination revealed extensive inflammatory cell influx in the BLT2<sup>−/−</sup> mice receiving WT marrow. Relatively greater inflammation was observed in WT mice receiving WT bone marrow compared with the naive mice (Figs. 3E, 4E). This could be due to the fact that the data in Fig. 4 were collected at day 14 of arthritis, whereas the sections in Fig. 3 were generated from mice at day 22 of arthritis. The more severe bone loss in mice used in the bone marrow-transplant experiments could be related to the age and/or to exposure to radiation. Bone marrow-recipient mice were ∼4–6 wk older than naive mice as a result of the delay to ensure full engraftment.

Because WT and BLT2<sup>−/−</sup> mice express similar levels of BLT1, it is clear that expression of BLT1 alone in the BLT2<sup>−/−</sup> mice is not sufficient for arthritis induction. As noted earlier, in K/BxN arthritis, it was demonstrated that the first responding neutrophils must express BLT1 (9). These neutrophils produce copious amounts of LTB<sub>4</sub> (31), and the recruitment of additional neutrophils does not require BLT1. Indeed, at these high concentrations of LTB<sub>4</sub>, BLT1 may be downregulated by ligand-induced internalization (34). This implicates BLT2, the low-affinity LTB<sub>4</sub> receptor; however, the apparent lack of expression in murine neutrophils and the discovery of the high-affinity 12-HHT for BLT2 draws into question whether LTB<sub>4</sub> is an important ligand of BLT2 in vivo. Several lines of evidence help to clarify this issue. We recently published results demonstrating that a lack of 12-HHT synthetic capacity does not impede arthritis development or severity in the K/BxN serum-transfer model (35). Further, our experiments with 300.19 cells expressing BLT1 and BLT2, as well as with synovial neutrophils, clearly show that 12-HHT is a weak or partial agonist in vivo; therefore, LTB<sub>4</sub> could have a significant role in mediating biological activities of BLT2. It seems that 12-HHT is unable to fully activate BLT2, whereas LTB<sub>4</sub> is a more
robust, albeit low-affinity, ligand for BLT2. It also seems that synovial neutrophils do not express BLT2 at all or preferentially use BLT1 for chemotaxis. Whereas the present studies clearly outline an important role for BLT2 in inflammatory arthritis, the cell type responsible for mediating this effect remains elusive. Although no changes were observed in steady-state BLT1 expression in neutrophils from BLT2Δ/Δ mice, we cannot rule out alterations in BLT1 expression under conditions of induced inflammation in the joints.

In summary, the results presented in this article suggest a novel role for BLT2 in arthritis. While acknowledging the fact that mouse models might not reflect arthritis development or progression in humans, consistent results for the role of the LTB4 pathway in various mouse models of arthritis suggest a re-evaluation of this pathway as a target for human RA. The results of this study demonstrate that the concentration of the antagonist must be optimized to be able to properly block signaling through BLT1 or BLT2. Given the apparent role of BLT2, it is important to identify the cell types that express BLT2 in human arthritis and to study the interplay between the two ligands LTB4 and 12-HHT. Because BLT1 and BLT2 are structurally similar, targeting these two receptors with highly specific dual inhibitors might offer an attractive treatment option for human RA.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. Reconstitution of bone marrow recipient mice. Peripheral blood was stained with murine CD45.1-PE and CD45.2-FITC and gated on PMNs by forward and side scatter. PMNs from WT mice receiving WT bone marrow (a), BLT2-/- mice receiving WT bone marrow (b) and WT mice receiving BLT2-/- bone marrow (c) were analyzed for the percentage of cells in the periphery from donor origin. All mice were found to have at least 94% of peripheral cells originating from donor origin.