Targeted Disruption of Leukotriene B_4 Receptors BLT1 and BLT2: A Critical Role for BLT1 in Collagen-Induced Arthritis in Mice[1]

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Leukotriene B_4 mediates diverse inflammatory diseases through the G protein-coupled receptors BLT1 and BLT2. In this study, we developed mice deficient in *BLT1* and *BLT2* by simultaneous targeted disruption of these genes. The BLT1/BLT2 double-deficient mice developed normally and peritoneal exudate cells showed no detectable responses to leukotriene B_4 confirming the deletion of the *BLT1/BLT2* locus. In a model of collagen-induced arthritis on the C57BL/6 background, the BLT1/BLT2$^{-/-}$ as well as the previously described BLT1$^{-/-}$ animals showed complete protection from disease development. The disease severity correlated well with histopathology, including loss of joint architecture, inflammatory cell infiltration, fibrosis, pannus formation, and bone erosion in joints of BLT1/BLT2$^{+/+}$ animals and a total absence of disease pathology in leukotriene receptor-deficient mice. Despite these differences, all immunized BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ animals had similar serum levels of anti-collagen Abs relative to BLT1/BLT2$^{+/+}$ animals. Thus, BLT1 may be a useful target for therapies directed at treating inflammation associated with arthritis. *The Journal of Immunology,* **2006, 176: 6254–6261.**

Rheumatoid arthritis (RA)[3] is a chronic inflammatory disease involving multiple joints and remains an autoimmune disease of unknown etiology (1). Collagen-induced arthritis (CIA) is a model for RA that is induced in susceptible mouse strains by intradermal immunization with collagen type II (CII) emulsified in a complete adjuvant (2, 3). The significance of this model is that CII is the major constituent protein of cartilage in diarthrodial joints, the predominant site of inflammation in RA. In addition, the pathogenesis of CIA is in many ways similar to that of RA as both RA and CIA are characterized by an intense synovitis accompanied by erosions of cartilage and subchondral bone by a pannus-like tissue (4). Susceptibility to CIA was considered to be MHC class-linked (H-2^q and H-2^r) as only DBA/1 (H-2^q) and B10.RIII (H-2^r) mice, among the most commonly used strains, are susceptible to CIA (5). However, recently Campbell et al. (6, 7) modified the immunization procedure and showed that clinically and histologically similar CIA may be induced in C57BL/6 (B6) mice. In addition to serving as a valuable tool to study immunity to CII, the CIA model has proven equally useful to investigate inflammatory joint injury and led to the development of novel TNF-based therapies for human RA (8, 9).

Leukotriene B_4 (LTB_4; (5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicostatetraenoic acid) is one of the most potent chemoattractants of leukocytes (10). LTB_4 promotes inflammation by stimulating CD11b up-regulation and adhesion of leukocytes, emigration of leukocytes from the bloodstream, neutrophil activation leading to respiratory burst, degranulation, and release of enzymes (10). In addition, LTB_4 can alter transcriptional profiles resulting in proinflammatory amplification circuits (11, 12). These processes have been implicated in the pathogenesis of a variety of diseases such as atherosclerosis, asthma, allergic encephalomyelitis, psoriasis, and inflammatory bowel disease (13, 14). A role for LTB_4 in RA was suggested by several observations over the past two decades. LTB_4 levels in synovial fluids from patients with active RA were 5-fold higher relative to synovial fluids from osteoarthritis (15, 16). Neutrophils from RA patients undergoing methotrexate therapy displayed both acute and chronic suppression of LTB_4 synthesis ex vivo (17). LTB_4 receptor antagonists were found to inhibit CIA in mice (18, 19). Mice deficient in 5-lipoxygenase-activating protein (FLAP), and as a consequence in LTB_4 synthesis, were partially protected from developing CIA (20).

Two distinct G protein-coupled receptors, BLT1 and BLT2, likely mediate the effects of LTB_4 in different cell types (21, 22). BLT1 is a high-affinity receptor expressed in a variety of leukocytes including neutrophils, monocyte/macrophages, eosinophils, mast cells, and activated T lymphocytes. BLT2 is a low-affinity LTB_4 receptor more widely expressed in human tissues. Of interest, high levels of BLT2 mRNA expression were observed in actively inflamed synovial tissue from patients with RA where as leukocytes infiltrating synovial fluid predominantly expressed BLT1 mRNA in these patients (23). Previously described BLT1$^{-/-}$ mice allowed the determination of a critical role for BLT1 in atherosclerosis and airway hyperresponsiveness (11, 24, 25). However, the physiological role of BLT2 is unknown. The genes for BLT1 and BLT2 are adjacent to each other in both mouse and human genomes and are separated by only 4 kb of intergenic region. Moreover, the promoter of the *BLT1* gene is within the coding region of BLT2 (26).

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, collagen type II; LTB_4, leukotriene B_4; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; ES, embryonic stem; m, murine; CIDE-B, cell death-inducing DFF45-like effector B; ORF, open reading frame; PAF, platelet-activating factor; COX2, cyclooxygenase 2; WT, wild type.

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Because crossing of single knockout animals cannot be used to create a double knockout of BLT1 and BLT2, we have generated BLT1/BLT2 double-deficient mice by directly targeting both receptors. In this study, we tested the $BLT1^{-/-}$ and the $BLT1/BLT2^{-/-}$ mice in the CIA model. The results showed that disruption of BLT1 alone is sufficient to offer complete protection of mice from developing arthritis, whereas anti-CII Ab levels in $BLT1^{-/-}$ and $BLT1/BLT2^{-/-}$ were similar to the $BLT1/BLT2^{+/+}$ mice.

Materials and Methods

Targeting construct and generation of BLT1/BLT2 double-deficient mice

The previously described BLT1 targeting construct was modified to target both *BLT1* and *BLT2* genes (27). The BAC clone containing the mouse *BLT1* and *BLT2* genes was mapped by restriction analysis, and a 4.5-kb fragment 10 aa upstream of the BLT2 coding region was subcloned into the *Not*I-*Xho*I sites of the pBluescript vector. The EGFP expression cassette was PCR amplified from pEGFP-N2 (BD Clontech) and inserted in-frame after the first 10 aa of the BLT2 coding region at the *Xho*I site of the 4.5-kb fragment. This fragment with EGFP included was used to replace the long arm of the previously described targeting construct (27). The resulting final construct pWSGB was 16 kb in length. The mock construct which served as a positive control for PCR screening of the embryonic stem (ES) cell clones was the same as previously described (27). AK7 (129S4/SvJaeSor) ES cells (10^7) were electroporated with 25 μg of *Not*I-linearized WSGB-DNA. The transfected cells were grown in DMEM with 200 μg/ml G418 and 2×10^{-6} M ganciclovir for 10 days. Surviving clones were tested for recombination using a neomycin-coding sequence primer—tcgcagcg-catcgccttctatcg—and a primer from the 3′ end of *BLT1* gene external to the knockout construct—gctgggagtcatcaacaagcactc. Of the eight positive clones, four were expanded and the genotypes were confirmed by Southern blot analysis with 10–20 μg of DNA using the 0.9-kb probe (*Bgl*II/*Bam*HI fragment) external to the 3′ end of the knockout construct. Two undifferentiated clones were individually microinjected into C57BL/6J blastocysts and transferred into pseudopregnant C57BL/6 mice. Chimeric mice generated from two individual cell 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 using Southern blotting with the same probe indicated above or more routinely using a three primer PCR with the primers 1) atgtctgtctgctaccgtcc, 2) aggtgcagca caagtgtggc, and 3) cagctcgaccaggatggg. All mice were housed in a specific pathogen-free barrier facility. Mice were 8–12 wk old at the time of use. All studies and procedures were approved by the Animal Care and Use Committee of University of Louisville Research Resources Center.

Generation and analysis of 300.19 cell lines expressing murine BLT1 and BLT2

Murine (m) BLT1 and BLT2 were stably expressed to similar levels in a mouse pre-B cell line, 300.19 (28). Hemagglutinin-tagged mBLT1 or mBLT2 cDNAs (20 μg) in the eukaryotic expression vector pRK-5 were transfected into 300.19 cells by electroporation, selected for G418 resistance, and stained with 12CA5 Ab and cells expressing the hemagglutinin epitope on the surface were sorted by flow cytometry. Cells expressing similar levels of the receptor were analyzed for functional receptor expression. For calcium mobilization, 3×10^6 cells were washed and loaded with 1.0 μM Indo-1 AM for 30 min at 37°C as previously described (29). 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. Calcium mobilization in 4-h zymosan A-elicited peritoneal lavage cells containing over 80% neutrophils was also measured essentially by the same procedure.

Northern blot analysis

Mouse neutrophils and macrophages were isolated from the peritoneal lavage after 4 and 72 h of zymosan A treatment, respectively. Total RNA isolation was performed as described according to the manufacturer's directions (RNeasy; Qiagen). Twelve micrograms of total RNA from mouse spleen, liver, neutrophils, and macrophages were denatured, electrophoresed on 1.2% formaldehyde-agarose gel, and transferred to nylon membranes (Hybond-N^+; Amersham Biosciences). The membranes were hybridized with ^{32}P-labeled open reading frame (ORF) of mouse BLT1, BLT2, cell death-inducing DFF45-like effector B (CIDE-B), and β-actin at 42°C overnight in an ULTRAhyb hybridization buffer (Ambion). The membranes were washed in 2× standard saline citrate phosphate/EDTA, 0.1% SDS, followed by washing in 0.1× standard saline citrate phosphate/EDTA, 0.1% SDS at 55°C for 1 h, and subjected to autoradiography.

Mice used in CIA experiments

The previously described $BLT1^{-/-}$ mice (27) have been backcrossed onto the B6 background for seven generations and the experimental control mice for this group were purchased from The Jackson Laboratory. The $BLT1/BLT2^{+/-}$ mice were backcrossed onto B6 for nine generations. The $BLT1/BLT2^{+/-}$ mice were then intercrossed to generate three groups of $BLT1/BLT2^{+/+}$, $BLT1/BLT2^{+/-}$, and $BLT1/BLT2^{-/-}$ littermate mice. All mice were >8 wk of age at the time of experimentation, and were age matched.

Induction and assessment of arthritis

CFA was prepared by mixing 100 mg of heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories) in 20 ml of IFA (Sigma-Aldrich) (6). An emulsion was formed by dissolving 2.0 mg/ml chick CII (CII; Sigma-Aldrich) overnight at 4°C in 10 mM acetic acid and combining it with an equal volume of CFA. CII solution and the emulsion with CFA were always freshly prepared. Mice were injected i.d. at the base of the tail with a total of 100 μl of emulsion containing 100 μg of CII and 250 μg of *M. tuberculosis*. The same injection was repeated at day 21; however, due to toughening of the skin at the base of the tail, booster injections were distal to the primary injection site.

Clinical and histological assessment of arthritis

All mice were examined two to three times per week for the initial visual appearance of arthritis after immunization. Arthritis of each individual limb was graded using the following scoring system: 0, normal; 1, apparent swelling and redness limited to individual digits; 2, swelling in more than one joint; 3, severe redness and swelling of the entire paw including digits; and 4, maximally inflamed limb with involvement of multiple joints. The maximum score per mouse was 16. Mice were scored as arthritic if more than one paw had a score >2. The thickness of the hind paws was measured using a dial gauge caliper (Mitsutoyo). At the end of the experiment, the rear paws and joints were removed, fixed, decalcified, and paraffin embedded. Joint sections (5 μm) were stained with H&E and examined for the histological changes of inflammation, pannus formation, cartilage, and bone damage. Arthritic changes in the ankle were scored as previously described: 0, normal; 1, weak leukocyte infiltration but no erosion; 2, modest infiltration and weak erosion; 3, severe infiltration and invasion of bones; and 4, loss of bone integrity (30).

Determination of serum anti-collagen Ab levels by ELISA

Blood was collected by cardiac puncture. After clotting at room temperature for 1 h, the samples were kept overnight at 4°C and the serum was collected by centrifuging at 13,000 rpm for 5 min. ELISAs for Abs to CII were performed as described in the Arthrogen-CIA manual (Arthrogen-CIA kit; Chondrex). In brief, precoated plates were washed and incubated with blocking buffer for 2 h at room temperature. Serum at a 1/37,500 dilution was added to each well. Plates were incubated at 4°C overnight, washed six times and incubated with peroxidase-conjugated goat anti-mouse IgG for 2 h at room temperature, and then washed six times. Peroxidase activity was then determined following the addition of orthophenylenediamine chromagen in urea H_2O_2 buffer for 30 min by determining the OD at 490 nm. Each sample was tested in duplicate and the mean value was recorded.

Immunohistochemistry

Immunohistochemistry was performed on deparaffinized slides using the traditional primary/secondary Ab-peroxidase technique. Briefly, paraffin-embedded sections were dewaxed and hydrated, Ag was retrieved with Tris/EDTA buffer, and endogenous peroxidase was blocked by incubating with 0.3% H_2O_2 for 10 min. Sections were then block with 3% BSA and following with primary Ab and corresponding HRP-secondary Ab staining. Color was developed by treatment with 3,3′-diaminobenzidine (Sigma-Aldrich) and sections were counterstained with hematoxylin.

Results

mBLT2 is a functional LTB_4 receptor

Although the *BLT2* gene has been identified from several sources, the functional activity of BLT2 in primary cells is yet to be demonstrated. To determine whether the mBLT2 is a functional LTB_4

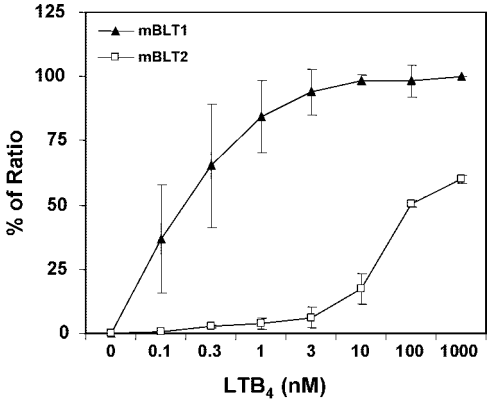

FIGURE 1. mBLT2 is a functional LTB_4 receptor. The 300.19 cells expressing the mBLT1 or mBLT2 were loaded with Indo-1 and release of intracellular calcium was measured as described in *Materials and Methods*. Response to increasing concentrations of LTB_4 was monitored in real time. Dose-response curves of percent calcium release for mBLT1 and mBLT2 were shown. Data represent average measurements from three different calcium traces for each concentration.

receptor, we generated stable 300.19 cell lines expressing this receptor. Fig. 1 shows dose-response profiles of LTB_4-induced calcium mobilization in cells expressing mBLT1 or mBLT2. Consistent with the results from cell lines expressing human BLT1 and BLT2, these cells activated dose-dependent calcium release with mBLT1 showing at least 10-fold more sensitivity to LTB_4 than mBLT2.

Generation of BLT1/BLT2 double-deficient mice

To delete both BLT1 and BLT2 genes simultaneously, we used a modified targeting vector from the one used to generate BLT1-deficient mice (27). In this vector, the entire region between amino acid no. 10 of BLT2 through amino acid 316 of BLT1 was deleted and replaced with the PGK-neomycin (PGK-Neo) cassette (Fig. 2*a*). In addition, the sequence encoding an in-frame fusion of the GFP coding region was inserted following codon no. 10 of BLT2. BLT1/BLT2$^{-/-}$ mice were generated essentially following the same protocols described for BLT1$^{-/-}$ mice (27). Fig. 2*b* shows a Southern blot of *Bam*HI- and *Xho*I-digested DNA from the three genotypes. The BLT1/BLT2$^{+/+}$ littermate lanes (+/+) show the expected 5.3-kb band and the homozygous lanes (−/−) show the 6.5-kb mutant band. The heterozygous (+/−) lanes show both the wild-type (WT) and mutant bands. BLT1/BLT2$^{-/-}$ mice were born at the expected Mendelian ratios and showed no overt developmental or morphological abnormalities. Three primer PCRs to identify the junction of the BLT2 and GFP fusion in the double-deficient mice (Fig. 2*c*, *left*) or the neo gene and BLT1 at the 3′ end (Fig. 2*c*, *right*) were routinely used to determine the genotypes from the genomic DNA isolated from tail biopsies.

Analysis of lymphoid tissues found no gross alterations in the size of the thymus, spleen, or lymph nodes between BLT1/BLT2$^{-/-}$ and WT littermates. The number and distribution of CD4$^+$ and CD8$^+$ T lymphocytes, or B220$^+$ B lymphocytes found within the spleen, peripheral lymph nodes, mesenteric lymph nodes, or within the blood were similar in BLT1/BLT2$^{-/-}$ and control animals (data not shown). In addition, no significant differences were found in numbers of circulating lymphocytes, monocytes, neutrophils, or eosinophils or in serum IgG and IgM levels between the BLT1/BLT2$^{-/-}$ and BLT1/BLT2$^{+/+}$ mice (data not shown).

Analysis of mBLT2 *gene expression*

To confirm that the mutation disrupted the LTB_4 receptor expression and not other chemoattractant receptors, zymosan-elicited peritoneal exudate cells (over 80% neutrophils) were analyzed for calcium mobilization. Both LTB_4 and platelet activating factor (PAF)-induced calcium mobilization in cells from littermate BLT1/BLT2$^{+/+}$ animals (Fig. 3*a*). In contrast, cells from the BLT1/BLT2$^{-/-}$ animals showed no calcium mobilization in response to LTB_4 but equivalent responses to PAF compared with cells from BLT1/BLT2$^{+/+}$ mice.

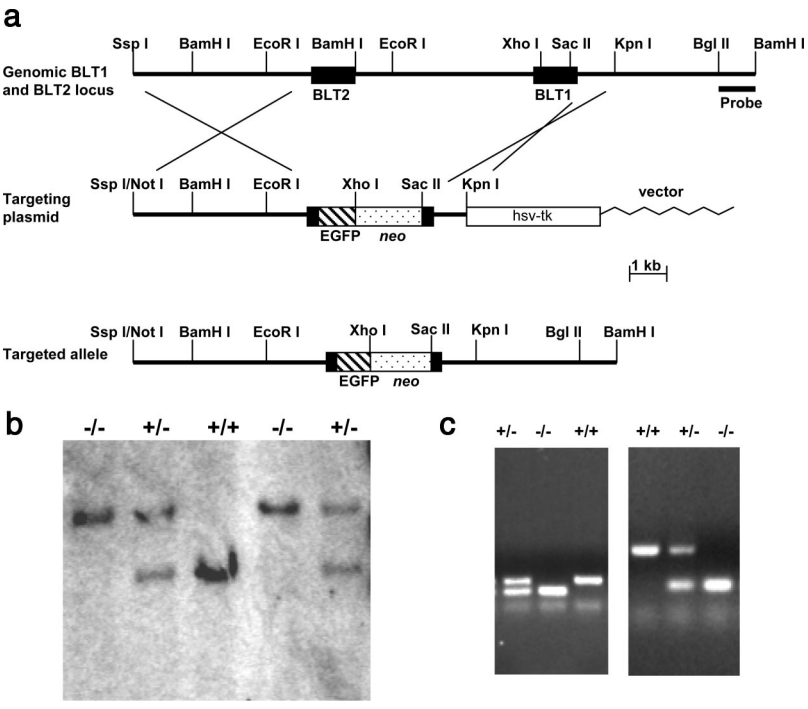

FIGURE 2. Targeted disruption of mouse *BLT1/BLT2*. *a*, Genomic locus of *BLT1* and *BLT2*, targeting vector, and the recombinant mutant genomic locus. Coding region of the *BLT1* and *BLT2* gene is indicated as solid boxes. Six kilobases of the coding region and the untranscribed region between BLT1 and BLT2 ORF was replaced with PGK-*neo* cassette in the targeting vector. An enhanced GFP (EGFP) expression cassette was inserted in-frame 30 bp downstream of BLT2 ORF and upstream of the *neo* gene. The final construct contained homology arms of 4.5 and 1.5 kb. A *Bgl*II-*Bam*HI fragment served as an external probe for Southern blot analysis of genomic DNA from ES cells and mouse tails. *b*, Southern blotting showing correct targeting and germline transmission of the mutated *BLT1/BLT2* gene. Genomic DNA samples prepared from F_2 offspring were digested with *Bam*HI and *Xho*I, separated on 0.75% agarose gels, blotted onto nylon membranes, and hybridized with the ^{32}P-labeled, 0.9-kb *Bgl*II-*Bam*HI fragment. The genotypes of the mice are indicated above the lanes. *c*, A three-primer PCR was designed to identify the WT, heterozygous, and homozygous mutant alleles at the BLT2/GFP junction (*left panel*). The previously described three-primer PCR methods used for screening BLT1-deficient mice was also run occasionally to confirm the genotype (*right panel*). The PCR and Southern blotting methods gave the same in all cases tested.

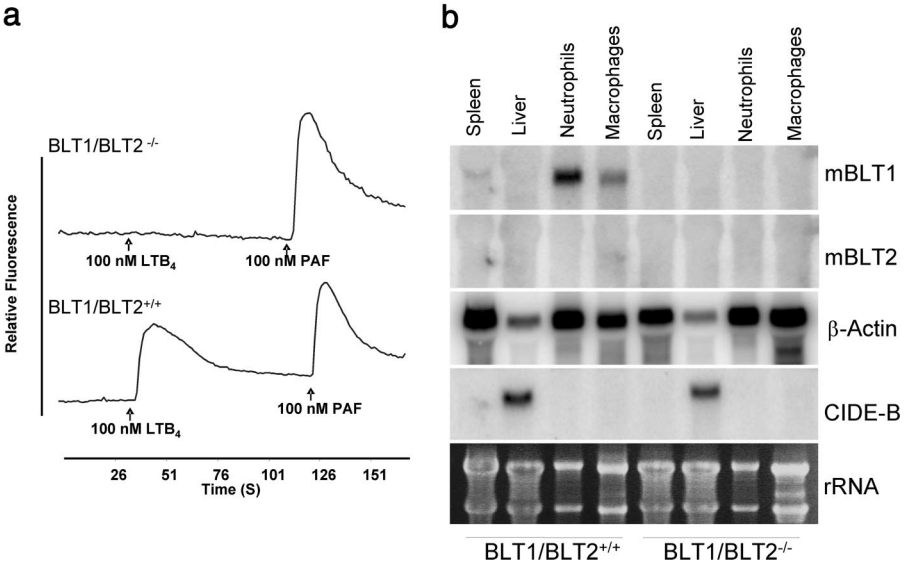

FIGURE 3. Expression analysis of mBLT2. *a*, Calcium mobilization of peritoneal neutrophils from BLT1/BLT2$^{+/+}$ and BLT1/BLT2$^{-/-}$ mice. Calcium flux was monitored in Indo-1-loaded, zymosan-elicited peritoneal neutrophils stimulated with 100 nM LTB_4 and 100 nM PAF as indicated. Each tracing represents an analysis of 3×10^6 cells from a single mouse with the indicated genotype, and the data shown is representative of at least three each of BLT1/BLT2$^{+/+}$ or BLT1/BLT2$^{-/-}$ animals. *b*, RNA blot analysis of mBLT1 and mBLT2. Fifteen micrograms of total RNA was isolated from spleen, liver, peritoneal neutrophils, or macrophages were separated on 1.0% agarose gels and transferred to nylon membranes. The RNA blot was sequentially hybridized with BLT1, BLT2, β-actin, and CIDE-B cDNA probes, and exposed to x-ray film for 3 h to overnight after washing. *Bottom*, Ethidium bromide-stained agarose gel shows comparable RNA loading in each lane.

Northern blot analysis of RNA from spleen, liver, peritoneal neutrophils, and macrophages showed strong expression of mBLT1 in neutrophils, and relatively lower levels of expression in macrophages in the BLT1/BLT2$^{+/+}$ mice, but no expression in the BLT1/BLT2$^{-/-}$ mice (Fig. 3*b*). Data also showed weak BLT1 expression in the spleen of BLT1/BLT2$^{+/+}$ mice (Fig. 3*b*). Hybridization with the mBLT2 ORF probe (Fig. 3*b*) or BLT2 5′-UTR or 3′-UTR (data not shown) failed to show any mBLT2 expression in any of the tissues studied. An overlapping divergently transcribed gene, *CIDE-B* in this locus showed normal liver-specific expression in both the BLT1/BLT2$^{+/+}$ and BLT1/BLT2$^{-/-}$ mice (Fig. 3*b*).

Although BLT2 was expressed in a wide variety of tissues, including liver and spleen in humans, we did not detect any GFP expression in these tissues either in the BLT1/BLT2$^{+/-}$ or in BLT1/BLT2$^{-/-}$ mice. Although flow cytometry revealed weak GFP expression in platelets, we could not detect any functional activity of BLT2 in these or other cells (data not shown).

BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice are completely resistant to CIA

Mice of the H-2^q (DBA/1J) background are highly susceptible to CIA whereas mice of the H-2^b (B6) are resistant. However, modification of the immunization procedure results in high incidence of CIA in B6 background mice (6). In two separate preliminary experiments, using the same method, we successfully induced arthritis in B6 strain mice (data not shown). To determine the role of LTB_4 receptors in arthritis, we set up two different study groups: the BLT1$^{-/-}$ mice were set up together with control B6 (BLT1/BLT2$^{+/+}$) mice and the littermate offspring from BLT1/BLT2$^{+/-}$ breeders (BLT1/BLT2$^{+/+}$, BLT1/BLT2$^{-/-}$, and BLT1/BLT2$^{+/-}$) constituted a second group. The arthritis symptoms in BLT1/BLT2$^{+/+}$, BLT1$^{-/-}$, BLT1/BLT2$^{-/-}$, and BLT1/BLT2$^{+/-}$ mice were studied after immunization with CII on day 0 and a boost with CII on day 21. Mice were examined weekly after the first immunization and every 2–3 days after the boost for signs of developing arthritis. The severity of the arthritis was assessed using a visual scoring system standardized under our laboratory experimental conditions (Fig. 4*a*). The BLT1/BLT2$^{+/+}$ animals developed clinical signs of arthritis with an incidence of 37 and 60% by 38 and 25 days, respectively (Fig. 4, *b* and *c*). Because male and female mice developed arthritis with comparable incidence, only the total number of mice is shown. The average cumulative clinical score and the swelling measured as Δpaw thickness are shown in Fig. 4, *d–g*. The clinical appearance of the swollen joints, the range of severity, and the progression to severe swelling was similar to that observed in DBA/1 mice. None of the BLT1$^{-/-}$ or BLT1/BLT2$^{-/-}$ developed any signs of arthritic disease as compared with the BLT1$^{+/+}$ control mice (Fig. 4). The intermediate incidence and severity of arthritis in the BLT1/BLT2$^{+/-}$ mice in this group indicated a possible gene dosage affect in the CIA model (Fig. 4, *c*, *e*, and *g*).

Histological features of immunized BLT1/BLT2$^{+/+}$, BLT1$^{-/-}$, and BLT1/BLT2$^{-/-}$ mice

An observer unaware of the genotype of the animals scored the histopathology of hind limb knee joints. The severity of disease as determined by the histological features correlated with the observed visual scores (Figs. 5, *a* and *b*, and 6). None of the BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice had any evidence of arthritis upon histological examination (Figs. 5, *c–f*, and 6). Pannus formation, fibrillation of the articular surface, and eventual ankylosis are hallmarks of RA. Mild to moderate pannus and fibrillation of the articular surface were common in the BLT1/BLT2$^{+/+}$ mice (Fig. 5, *g–i*). In contrast, none of the BLT1$^{-/-}$ or BLT1/BLT2$^{-/-}$ mice showed any sign of pannus formation or fibrillation of the cartilage (data not shown).

Immune response against CII in BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice

A high level of anti-CII Ab generation accompanies the development of disease in the CIA model (3). To investigate whether the disease-free incidence of arthritis in BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice was due to the lack of an Ab response to type II collagen, the anti-CII-specific levels of IgG in the serum were determined at the termination of the CIA experiment. Anti-CII Ab levels were similar in BLT1$^{+/+}$ mice and in BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice (Fig. 6).

Inflammatory cell infiltration in B6-CIA synovitis

To determine the type and extent of inflammatory cell infiltration occurring during disease development, histopathological examination and immunohistochemical staining of synovial tissues were performed. Fig. 7 shows the typical inflammatory cell infiltration found in BLT1$^{+/+}$ animals and complete absence of these cells in the BLT1$^{-/-}$ animals. Examination of arthritic tissue sections at

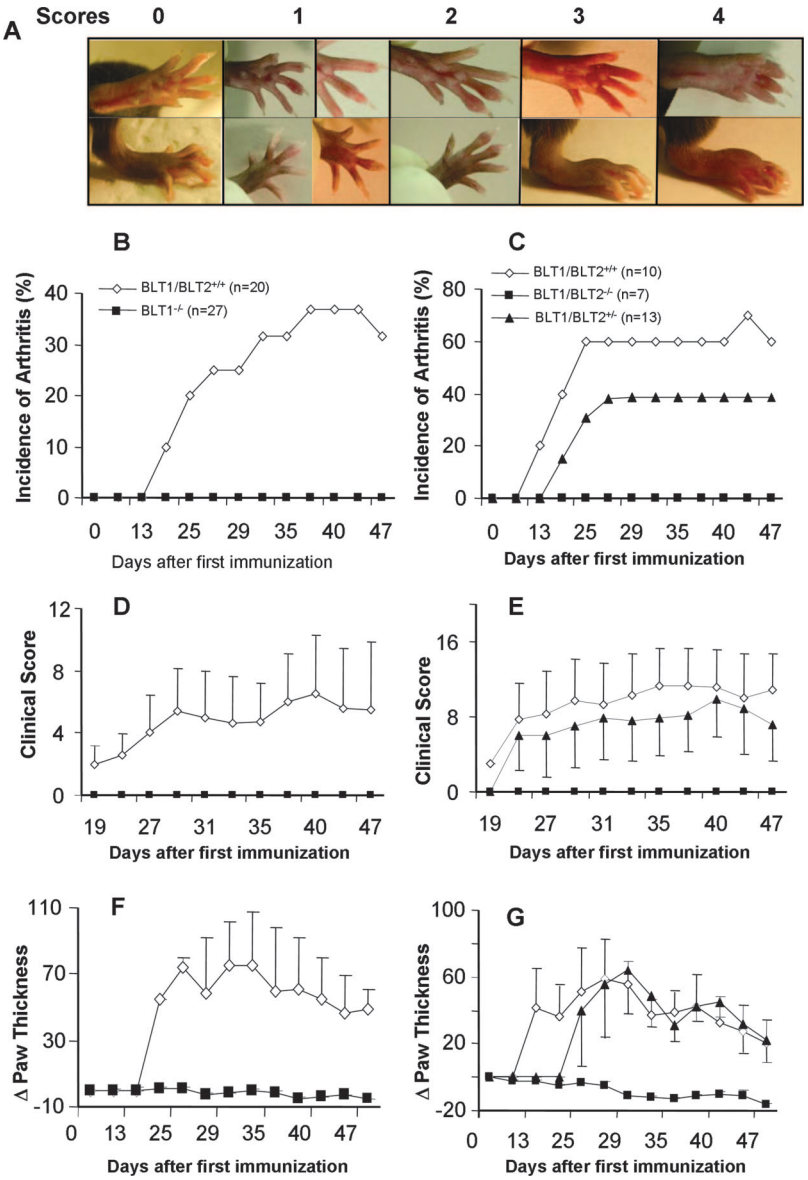

FIGURE 4. Resistance of BLT1$^{-/-}$ mice and BLT1/BLT2$^{-/-}$ to CIA. Age- and sex-matched B6 mice were immunized with chicken CII emulsified in CFA at day 0 and boosted at day 21. Mice were scored blind to the knowledge of the genotype of the animals every 2–3 days. *a*, Representative assignments of clinical scores for CIA-B6 model. *b* and *c*, Incidence of arthritis: mice were considered arthritic when inflammation was observed in any paw with clinic score >2, and number of arthritic mice in each group is shown as a percentage. *d* and *e*, Clinical score of arthritis: paws from each mouse were scored every 2–3 days. The scores from four paws were then combined for each mouse, and total severity score for the group was divided by the number of arthritic mice to obtain an average severity score. *f* and *g*, ΔPaw thickness: hind paw swelling was evaluated with spring-loaded caliper during the course of the disease. Average change in paw thickness increase obtained for each mouse by subtracting the baseline of paw thickness before the first immunization. SD is indicated with error bars.

higher magnification showed a predominant neutrophil-based inflammation (Fig. 7*b*) but also some lymphocytes (arrows) and macrophages (arrowhead). Immunohistochemical analysis with Gr-1 and CD3 Abs confirmed these observations (Fig. 8), whereas the BLT1$^{-/-}$ mice showed no detectable immunostaining with any of these markers.

Discussion

The deletion of the *BLT1/BLT2* genes reported in this study reveals a potential role for these receptors in inflammatory arthritis. The BLT1/BLT2$^{-/-}$ mice were viable, developed normally, and displayed no overt behavioral or morphological defects. The number and development of leukocyte subpopulations were normal in BLT1/BLT2$^{-/-}$ mice. The Southern and PCR analysis of mice and the demonstration that these mice lacked the expression of a functional LTB_4 receptor in peritoneal lavage cells clearly establishes the generation of a leukotriene receptor double-deficient mouse line.

The studies reported here demonstrate that expression of mBLT2 in cell lines results in a low-affinity receptor that responds to LTB_4, suggesting that mBLT2 is indeed an LTB_4 receptor. Although BLT2 was identified several years ago, its expression pattern in different tissues remains unclear. Although macrophages and to some extent neutrophils were suggested to express BLT2 (22, 31) and BLT2 mRNA was detected in a wide variety of human tissues, the expression of BLT2 in mouse tissues is unknown. Tager and Luster (32) suggested they could not detect BLT2 expression in mice. Our results are consistent with these findings that BLT2 mRNA was not detectable in murine tissues by Northern blot analysis. An examination of relative abundance of human and murine leukotriene receptor cDNA clones in public expressed sequence tag databases is also consistent with this observation. Although human BLT1 and BLT2 are represented at similar abundance (43 and 41 clones, respectively), the mBLT1 is more frequently identified than mBLT2 (22 vs 3 clones). These observations suggest that while the *mBLT2* gene is indeed expressed, the level of transcription of *mBLT2* gene is very weak relative to human BLT2. Based on the design of the construct, we expected the GFP expression to represent the native BLT2 expression pattern. Although the absence of GFP expression in most mouse tissues in the knockout/knockin mouse generated here may not represent the true expression pattern of BLT2, it is consistent with the failure to detect any BLT2 mRNA. Further biochemical as well as functional

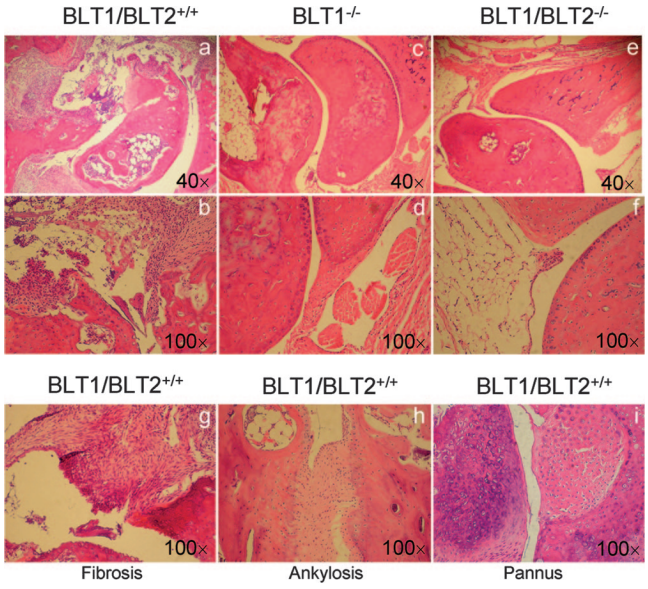

FIGURE 5. Histopathology of CIA in mice. Massive changes in the knee joints of the BLT1/BLT2$^{+/+}$ (*a* and *b*) but not in BLT1$^{-/-}$ (*c* and *d*) and BLT1/BLT2$^{-/-}$ (*e* and *f*) mice. Joint bones (tibia, tarsus), synovial joint tissue, and cartilage are shown. No histopathological changes in BLT1/BLT2$^{-/-}$ mice and BLT1$^{-/-}$ mice were observed. Histopathological changes of the WT mice were evaluated in terms of proliferation of synovial cells, necrosis/fibrosis (*g*), ankylosis (*h*), pannus (*i*), and the total severity of arthritic changes, including the destruction of cartilage and bone. Magnification is indicated at the *lower right corner* of each micrograph.

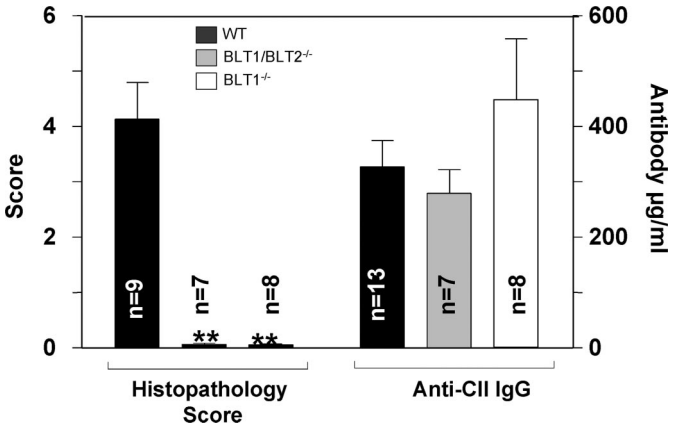

FIGURE 6. Histopathological scores and anti-CII IgG levels in CIA mice. BLT1/BLT2$^{+/+}$, BLT1$^{-/-}$, and BLT1/BLT2$^{-/-}$ mice are represented by ■, ▨, and □ columns, respectively. Histopathological evaluations were described in *Materials and Methods.* Both BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice produced comparable levels of anti-CII Abs relative to the WT mice both with and without disease, but the BLT1$^{-/-}$, and BLT1/BLT2$^{-/-}$ mice did not show any sign of disease both in clinical and histopathological views. The number of animals tested in each group is indicated.

studies with BLT2 single-deficient mice are needed to precisely define the mBLT2 function.

CIA has been the most widely used animal model for studying the pathogenesis of human RA and for screening novel therapeutic compounds. Although B6 mice are known to be resistant to CIA, it was the secondary but not the primary immune response to collagen that is defective in these mice (33). Campbell et al. (6) first reported that CIA could be induced in B6 at an incidence approaching that of congenic DBA/1 mice using an altered immunization protocol. Following the same procedure, with increasing the CFA concentration and administering both the primary and boost injection i.d., we could induce 40–70% incidence of arthritis in B6 mice. It should be noted that this level of incidence is relatively low compared with 70–100% incidence routinely observed for CIA in the DBA strain. However, the severity scores reported here are comparable to most studies on arthritis in DBA mice (34). These data suggest that the method of immunization but not the MHC haplotype might be a critical determinant of CIA incidence in B6 mice. The precise mechanisms whereby immunization with CII leads to a chronic arthritis are not known; however, data have shown that the CIA model is absolutely dependent on B cells and is significantly dependent on CD4$^+$ T cell involvement (35–38). Our results of comparable levels of anti-CII Ab indicate the normal functioning of B cells in the BLT1$^{-/-}$ and BLT1/BLT2$^{-/-}$ mice. It should also be noted that levels of anti-CII Abs in the WT animals also did not correlate with the disease incidence i.e., most animals had similar levels of anti CII Ab but only some (40–60%) did get the disease. This may be related to an activation event that requires BLT1. In this regard, the function of LTB_4 as a chemoattractant for activated CD4$^+$ and CD8$^+$ T cells expressing BLT1 (24, 39) may play an essential role in the B6-CIA model, because substantial numbers of CD3$^+$ cells were detected in the synovial tissue of the BLT1/BLT2$^{+/+}$ arthritic mice but not in the BLT1$^{-/-}$ mice.

Synovial fluid rich in inflammatory cells, in particular neutrophils, characterizes human RA (40). Neutrophils have been considered a key player in the articular and extra-articular manifestations of the disease. This appears to be the case for the CIA model in B6 mice as well. A variety of cells have been suggested as contributing to the initiation and progression of the immune response in rheumatoid synovium, including neutrophils, T cells, macrophages, fibroblasts, synoviocytes, and endothelial cells. Although the interplay between these cell types and the specific site of BLT1 activity remains to be established, lack of T lymphocytes in synovial cavity of the immunized BLT1 mice suggests an early role of BLT1 in disease development. Moreover, complete absence of neutrophil recruitment in BLT1$^{-/-}$ mice, and the known activity of BLT1 in neutrophils suggest that BLT1 could also play a direct role in neutrophil recruitment to the arthritic joints.

Multiple studies over the past two decades have suggested a role for arachidonic acid-derived lipid mediators in human RA (41, 42). Moreover, mice deficient in cyclooxygenase 2 (COX2), cytosolic phospholipase A2, and FLAP are all protected from CIA (20, 43–45). Although COX2 inhibitors were in extensive use for human RA treatment until recently, cardiovascular side effects led to a drastic reduction in use of these compounds (46, 47). Leukotrienes were recently demonstrated to be effective promoters of atherosclerosis (14). Inhibition of COX2 could result in increased arachidonic acid production, a substrate for 5-LO pathway leading to increased leukotriene generation that might account for the observed side effects of COX2 treatment. Thus, leukotrienes could offer an alternate target for treating inflammatory arthritis.

The current results of complete inhibition of CIA in BLT1-deficient B6 mice need to be considered in the context of several earlier studies using LTB_4 biosynthesis inhibitors or receptor antagonists in mice and in human RA clinical trials. First, several studies have shown that antagonists of BLT1 offer significant but not complete reduction of CIA development in mice (18, 19). In addition, in a model of IL-18 enhanced CIA in the DBA strain, a role for LTB_4 was established and inhibition of CIA induction by MK-886, a FLAP inhibitor, was observed (48). Moreover, in a DBA model of CIA partial protection from arthritis was observed in FLAP-deficient mice (20). The partial protection observed with

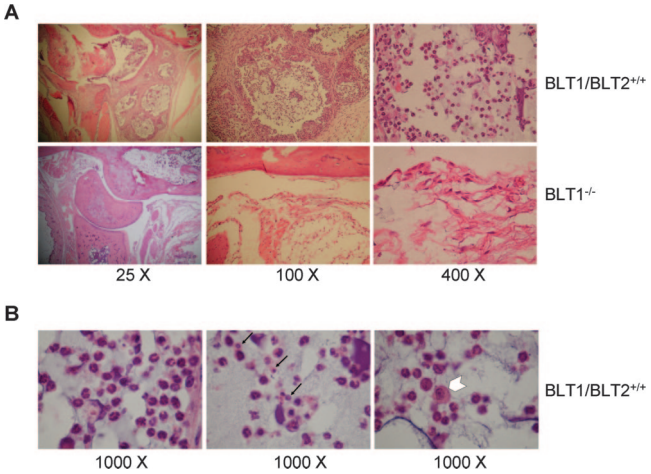

FIGURE 7. Inflammatory cell infiltration in the joint sections. *a*, Inflammatory cells infiltration in the synovial cavity in the joints of the WT mice but not the BLT1$^{-/-}$ joint. *b*, Higher magnification (×1000 original) showed the majority of the infiltration is neutrophils (*right*), with occasional lymphocytes (arrows) and macrophages (arrowhead) among the neutrophils (*middle* and *left*).

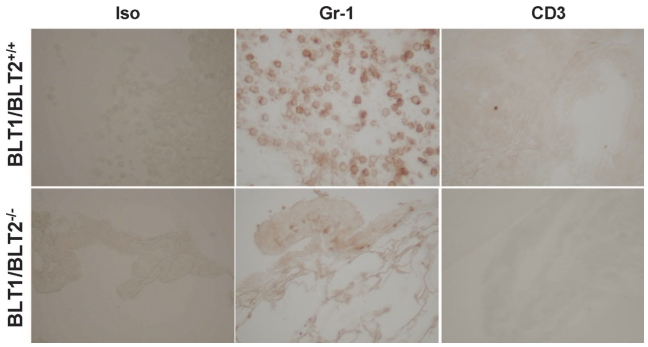

FIGURE 8. Immunohistochemistry staining of inflammatory cells in joint sections. Sections of CIA synovial tissues were stained with rat anti-mouse neutrophils Ab and rat anti-human CD3, which cross-reacts with mouse CD3. Correspondent isotype control was included as indicated. All sections were counterstained with H&E. All images shown are ×400 original magnification.

receptor antagonists as well other inhibitors and mouse models may be related to the incomplete targeting of the BLT1 and the differences in the DBA vs B6 model.

Several leukotriene antagonists and biosynthesis inhibitors have been used in clinical trials for human RA, but none have been approved for the treatment of RA (49, 50). Although the elements of inflammatory response are similar, murine CIA has limitations in modeling human arthritis, including the absence of rheumatoid factor as well as lack of clear mechanisms for the initiation of disease in humans (5). There are several potential explanations for the clinical failure of past leukotriene-based drugs for treatment of arthritis. Because all of the leukotriene antagonists are selected based on assays of neutrophil function, they may not have complete blocking activity on other cell types such as T cells. Another reason might be related to genetic variation in human populations. Although most of the inbred mouse models have shown strong leukotriene effects in CIA models, only subsets of human RA patients might benefit from the leukotriene antagonist-based therapies. Although the current study has shown complete protection against CIA in BLT1-deficient mice, the known expression pattern of BLT2 in human synovial tissues (23) suggests another potential difference in leukotriene involvement in human RA vs murine CIA. Despite these limitations, studies on RA patients might benefit from reinvestigation of dose, efficacy, and pharmacokinetics of the previously tested compounds as well as direct examination of the involvement of this pathway in human RA.

In summary, this study has described the generation of BLT1/BLT2$^{-/-}$ mice and defined a critical role for BLT1 in CIA. Because the loss of BLT1 alone is sufficient to offer complete protection against CIA, a role for BLT2 in this model could not be established. However, in a number of other models, including atherosclerosis and asthma, loss of BLT1 only offers partial protection or delays the progression of the disease (11, 25). The BLT1/BLT2$^{-/-}$ mice will be a valuable resource in further studies in these and other models of inflammatory diseases.

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Disclosures

The authors have no financial conflict of interest.

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