A unique requirement for the leukotriene B₄ receptor BLT1 for neutrophil recruitment in inflammatory arthritis

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Neutrophil recruitment into tissue plays an important role in host defense and disease pathogenesis, including the inflammatory arthritides. A multitude of diverse chemotactic signals have been implicated in neutrophil recruitment, suggesting that they have overlapping functions in mediating this critical biological response. However, here we demonstrate a unique, non-redundant role for the leukotriene B₄ receptor BLT1 in mediating neutrophil recruitment into the joint in the K/BxN mouse model of inflammatory arthritis. We demonstrate that neutrophil expression of BLT1 was absolutely required for arthritis generation and chemokine production in this model, and that specific BLT1 inhibition reversed established disease. Adoptive transfer of wild-type (WT) neutrophils restored arthritis and chemokine production in BLT1⁻/⁻ mice. Surprisingly, the primary effect of the transferred WT neutrophils into BLT1⁻/⁻ mice was to promote the entry of endogenous BLT1⁻/⁻ neutrophils into the joints of these mice. However, continued joint inflammation was dependent on the presence of WT neutrophils, indicating an ongoing specific requirement for BLT1-activated neutrophils in mediating BLT1⁻/⁻ neutrophil recruitment by other chemotacticants. These experiments demonstrate that neutrophil BLT1 functions in a novel and essential non-cell-autonomous manner to enable the recruitment of additional neutrophils not expressing this receptor, thereby amplifying the inflammatory response in autoantibody-induced arthritis.

Although the innate branch of the immune system is poised to protect the host in response to injury or infection, its inappropriate activation often leads to pathologic accumulation of leukocytes in affected organs. A diverse array of chemotactic signals is capable of recruiting leukocytes to sites of inflammation, including chemokines, bacterial peptides, proteolytic fragments of complement, and lipids. Of these mediators, leukotriene B₄ (LTB₄) is a highly potent lipid chemotactic produced and released within minutes by neutrophils, macrophages, and mast cells, positioning it uniquely as a key element of the immediate inflammatory response (1). LTB₄ binds with high specificity and affinity to BLT1, a G protein–coupled seven transmembrane–spanning receptor (2, 3), which is highly expressed on neutrophils and induces their chemotaxis and adhesion in response to LTB₄ (4, 5). Within the inflamed joints of patients with rheumatoid arthritis (RA), elevated levels of LTB₄ correlate with disease severity (6) and synovial fluid leukocytes highly express BLT1 (7), suggesting that this receptor–ligand pair contributes to the characteristic synovitis of RA by recruiting leukocytes into the inflamed joint.

The K/BxN serum transfer model of inflammatory arthritis bears certain clinical and histopathological similarities to human RA, including abnormal leukocyte accumulation in synovial tissue and fluid, synovial hypertrophy and pannus formation, and erosion of bone and cartilage. Transfer of serum from K/BxN transgenic mice containing autoantibodies against glucose 6-phosphate isomerase results in a robust polyarthritis (8) that is dependent on the orchestrated participation of key effectors of innate immunity, as the FcγRIII receptor, alternative complement pathway (9), and IL-1 (10) each play essential nonredundant roles in this
model. Innate immune cells are critically important, as mast cells (11) and neutrophils (12) are required for the generation of arthritis in this model. Although the identities and pathogenic importance of these leukocytes are now appreciated in this arthritis model, the specific chemotactic signals that guide these cells into the inflamed joint remain undefined. In these studies, we aimed to characterize the role of BLT1 in an antibody-induced model of arthritis to identify potential therapeutic targets and to understand the complex dynamics of leukocyte recruitment into the joint.

RESULTS AND DISCUSSION
BLT1 is required for the generation of autoantibody-induced arthritis
Because BLT1 is a potent mediator of leukocyte chemotaxis in the immediate innate immune response, we tested whether mice lacking BLT1 would be capable of developing arthritis. Although age-matched C57BL/6 WT controls developed polyarthritis within days of K/BxN serum injection, BLT1−/− mice remained largely disease free by measurable clinical parameters (Fig. 1, a and b). When BLT1−/− mice developed any joint swelling or erythema, it was limited to one portion of one joint and resolved within a few days. Histological analysis corroborated our clinical findings (Fig. 1 c), as joints of WT arthritic mice demonstrated characteristic inflammation, synovial hypertrophy, and joint erosions, findings that were absent or minimally present in BLT1−/− mice (Fig. 1 d). Therefore, despite the existence of multiple redundant chemoattractant pathways active upon leukocytes, we found an absolute requirement for BLT1 in inducing joint inflammation and destruction in this arthritis model.

We examined the therapeutic potential of inhibiting BLT1 in this model using CP-105,696, a potent, specific BLT1 antagonist that inhibits LTB4-induced neutrophil chemotaxis (13), using two different dosing strategies. The preventative dosing group received CP-105,696 before the induction of arthritis and was protected from developing disease, displaying very minimal joint swelling soon after serum injection that quickly resolved (Fig. 2, a and b). The therapeutic dosing group received CP-105,696 during very active arthritis, which halted progression of disease and reversed arthritis severity, although it did not remit completely (Fig. 2, a and b). Histological scoring demonstrated virtually no inflammation or bone and cartilage erosion when CP-105,696 was given preventatively and significant attenuation of these parameters when given therapeutically (Fig. 2 c). Additionally, although CP-105,696 effectively decreased synovial hyperplasia, BLT1 inhibition dramatically suppressed synovial neutrophil infiltration by 99.9 and 81.3% in the preventative and delayed treatment groups compared with the untreated group, indicating a primary role for BLT1 in recruiting neutrophils into the joint (Fig. 2 d). Although delayed inhibition of BLT1 was capable of limiting ongoing arthritis, its effectiveness as a protective agent suggests that BLT1 plays an important role in the early stages of disease. Protection by CP-105,696 in the K/BxN serum transfer model extends the therapeutic applicability of BLT1 antagonism, which also inhibited arthritis in a collagen-induced model (13).

BLT1 is necessary for synovial chemokine production in inflammatory arthritis
We next explored the mechanisms by which BLT1 may generate the appropriate signals for joint-specific inflammation. A very early step in the initiation of arthritis in this model is the rapid deposition of arthritogenic anti–glucose 6-phosphate isomerase autoantibodies upon cartilaginous and synovial surfaces of peripheral joints (8, 14), which is dependent on the presence of Fcγ receptor, neutrophils, and mast cells, but not the complement factor C5 (15). Although neutrophils have been shown to be necessary for joint-specific autoantibody localization (12), we found that both WT and BLT1−/− mice had similar levels of IgG lining the joint and cartilage surfaces, indicating that BLT1 does not play a role in...
the localization of arthritogenic antibody into the joint (Fig. 3 a). The fact that mice lacking functional BLT1 develop an early mild arthritis that quickly resolves after K/BxN serum injection (Figs. 1, a and b, and 2, a and b) suggests that BLT1 plays a critical role in inducing arthritis by amplifying the initial inflammatory response that follows arthritogenic antibody deposition within the joints.

To investigate the possibility that BLT1 mediates chemokine and inflammatory cytokine production, thereby amplifying subsequent inflammation, we measured their expression in the joints of WT and BLT1−/− mice using quantitative PCR (qPCR). In inflamed WT arthritic joints, levels of RNA for chemokines mediating neutrophil chemotaxis were elevated, notably MIP-2/CXL2 as well as MIP-1α/CCL3 and ENA-78/CXCL5, but not KC/CXCL1 (Fig. 3, b and c). Accordingly, levels of RNA for CXCR2 and CCR1, the primary murine neutrophil chemokine receptors, were elevated in the inflamed WT synovial fluid as well as the C5a receptor (C5aR), which mediates phagocyte chemotaxis toward the complement anaphylatoxin C5a (Fig. 3, b and c). Levels of RNA for CCR2 and CCR5, chemokine receptors commonly found on monocytes, were elevated in the inflamed WT synovial fluid (Fig. 3, d and e), but levels of CXCR3, the primary T lymphocyte chemokine receptor, were not. Elevated CXCR4 in WT joint fluid likely represents increased cellularity, as it is expressed on multiple cell lineages, and levels of IL-1β, a critical cytokine in the progression of K/BxN serum transfer arthritis (10), were significantly elevated, whereas TNF-α expression was not, consistent with the previous finding of this model’s variable dependence upon TNF-α (Fig. 3, b and c). However, when mice lacking functional BLT1 were used in these experiments, levels of all inflammatory mediators were consistently lower or absent (Fig. 3, b–e), indicating that BLT1 is needed for subsequent inflammation.

Figure 2. The BLT1 inhibitor CP-105,696 prevents and treats arthritis. The specific BLT1 inhibitor CP-105,696 was administered daily to C57BL/6 mice before the induction of disease on day −1 (preventative, filled arrows) or as disease was approaching maximal activity on day 5 (delayed, open arrows), and ankle thickness (a) and clinical score (b) were assessed daily (n = 4 in each group). Data are representative of three independent experiments. In a separate experiment, mice received sham gavage of vehicle lacking CP-105,696 without any effect on arthritis production (not depicted). (c) Histopathological scoring of ankles from control untreated mice compared with mice treated with preventative or delayed CP-105,696 dosing strategies (n = 10–15 in each group). Error bars represent SEM. (a) Total number of synovial cells and neutrophils within the joints of control untreated mice and mice treated with preventative or delayed CP-105,696 dosing strategies. *, P ≤ 0.005; **, P < 0.0001 (control vs. CP-105,696 treated).

Figure 3. BLT1 is required for synovial inflammatory chemokine and cytokine production in inflammatory arthritis. a) K/BxN serum was administered to WT and BLT1−/− mice, and ankles were harvested at 7 d. Frozen ankle sections were stained with FITC-labeled F(ab′)2 fragment directed against the Fc portion of mouse IgG. A WT mouse that did not receive any serum was used as a control. Bar, 230 μm. (b–e) Chemokine expression was measured by qPCR of total RNA isolated from synovial tissue on postserum transfer days 3 (early onset disease) (b) or 7 (early active disease) (c). Chemokine receptor expression was quantified by qPCR of total RNA isolated from synovial fluid at days 3 (d) and 7 (e). Total RNA was extracted from eight individual ankles in each experimental group, and qPCR reactions were run separately on each sample. Error bars represent SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.0005 (WT vs. BLT1−/−).
Neutrophil BLT1 directs leukocyte recruitment into the joint in a noncell–autonomous manner

Because neutrophils have been shown to be critical in inducing arthritis in the K/BxN serum transfer model of arthritis (12) and are present in high numbers in synovial fluid and at the cartilage–pannus junction in human RA, where they are capable of promoting significant joint inflammation and destruction (16), we wanted to determine whether the requirement for BLT1 is specific to the neutrophil population. Purified bone marrow neutrophils (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052349/DC1) were adoptively transferred to BLT1−/− mice on the same days of K/BxN serum transfer (days 0 and 2). All BLT1−/− mice that received WT neutrophils via adoptive transfer (WT PMN→BLT1−/− mice) subsequently developed arthritis (Fig. 4, a and b). Their disease course was nearly identical to the WT mice in the early stages, as disease was reaching maximal activity; however, the arthritis in WT PMN→BLT1−/− mice improved more rapidly than in the WT mice, completely resolving 1 wk earlier than the WT group. Synovial fluid analysis demonstrated similar numbers of neutrophils in both the WT and WT PMN→BLT1−/− mice at 20 h, but at 4 d, the number of synovial fluid neutrophils in the WT PMN→BLT1−/− mice was significantly lower (Fig. 4 c). Neutrophils have a short life span in the circulation of <24 h (17), so the attenuated disease course in the WT PMN→BLT1−/− mice demonstrates the requirement for the persistence of BLT1-expressing neutrophils for sustained joint inflammation. BLT1−/− mice that received BLT1−/− neutrophils via adoptive transfer did not develop arthritis and did not have transferred or endogenous BLT1−/− neutrophils in their joints, confirming that the effect of the neutrophil adoptive transfer was dependent on neutrophil BLT1 expression (Fig. 4, a–c).

In the WT PMN→BLT1−/− mice that developed arthritis, we examined whether BLT1-directed neutrophil trafficking into the synovial fluid was responsible for restoring joint inflammation. To do this, we transferred neutrophils from WT mice expressing the leukocyte antigen CD45.1+ to distinguish from host-derived CD45.2+ neutrophils by flow cytometry. Although one might have expected that the neutrophils in inflamed joints would be primarily the transferred WT neutrophils expressing BLT1 given their ability to recapitulate arthritis, we found that the vast majority of the synovial fluid neutrophils during peak disease activity (4 d) were actually endogenous BLT1−/− neutrophils (mean 93.4 ± 0.71% SEM) with very few transferred WT neutrophils (mean 6.6 ± 0.71% SEM; Fig. 4 d). When joint fluid was analyzed earlier at the initiation of clinical disease (20 h), transferred WT neutrophils constituted a greater proportion of total neutrophils (mean 38.66 ± 5.7% SEM; Fig. 4, d and e), indicating that the entry of a small number of WT neutrophils into the joint preceded the significant recruitment of BLT1−/− neutrophils into the joint during peak inflammation (Fig. 4 f). Levels of transferred WT neutrophils within the blood remained low at both time points (Fig. 4, d and e), indicating that the increased percentage of WT neutrophils in the joint at the early time point was not a mere reflection of the levels of WT neutrophils in the peripheral blood.

Adoptive transfer of WT neutrophils restored the upregulation of inflammatory chemokines in the joint (Fig. 4 g), thereby providing additional chemotactic signals for BLT1−/− neutrophils and other leukocytes to enter the joint and generate inflammatory arthritis. Interestingly, when arthritis trailed off in the WT PMN→BLT1−/− mice, synovial RNA levels of IL-1β and various chemokines, including those active on neutrophils such as ENA-78/CXCL5 and MIP-2/CXCL2, dropped off between the two groups (Fig. 4 f). These data suggest an ongoing requirement for BLT1 to maintain IL-1β and chemokine expression and subsequently recruit BLT1−/− neutrophils.

These experiments demonstrate that BLT1 expression by neutrophils is required for the development of inflammatory arthritis in the K/BxN serum transfer model by functioning in a non–cell-autonomous manner to allow the subsequent entry of neutrophils into the joint independently of BLT1. Furthermore, neutrophil BLT1 expression is required for the sustained production of IL-1β and multiple chemokines, which amplify the inflammatory response. Independent investigations have confirmed that neutrophils are also the primary source of synthesized LTB4 necessary for joint inflammation in this model, in addition to being the critical BLT1-expressing cell population (18). Collectively, these studies highlight the importance of an LTB4-BLT1 autocrine loop in conferring these neutrophils with the crucial function of directing cellular recruitment into the joint in a non–cell-autonomous fashion. Although BLT1 antagonism has been shown to be effective in inhibiting inflammation in another model of arthritis (13), our studies now describe an important new mechanism by which BLT1 induces joint inflammation.

Chemokines and other chemoattractants have long been noted to possess redundant roles in directing the trafficking of similar cell populations. However, we have now established the absolute, nonredundant requirement for BLT1 in the K/BxN serum transfer model of arthritis, in spite of the necessity of the C5a receptor (9) and up–regulation of multiple chemokines in this model. Previous studies have demonstrated that BLT1 activation can act synergistically with other chemoattractant receptors to promote leukocyte recruitment (19, 20). In this model, however, we have demonstrated that BLT1 works upstream of other chemoattractant receptors in a sequential manner as a mandatory event required for further leukocyte recruitment by other chemoattractants.

Upon activation, neutrophils release an array of proinflammatory products, including newly synthesized reactive oxygen metabolites, cytokines, chemokines, and lipids, as well as preformed granular enzymes and proteins (16, 21), and LTB4 is known to be a potent neutrophil secretagogue (22). We hypothesize that specific LTB4-induced BLT1 activation...
Figure 4. WT BLT1-expressing neutrophils are required for the generation of arthritis by promoting entry of BLT1<sup>−/−</sup> neutrophils into the joint. Bone marrow neutrophils from WT and BLT1<sup>−/−</sup> mice were adoptively transferred to BLT1<sup>−/−</sup> mice on days 0 and 2, and (a) ankle thickness and (b) clinical score were assessed daily. <i>n</i> = 3–4 in each group, and data are representative of three independent experiments. (c) Total numbers of Gr-1<sup>+</sup> neutrophils within the ankle synovial fluid of WT mice, BLT1<sup>−/−</sup> mice that received WT neutrophils (WT PMN→BLT1<sup>−/−</sup>), BLT1<sup>−/−</sup> mice, and BLT1<sup>−/−</sup> mice that received BLT1<sup>−/−</sup> neutrophils (BLT1<sup>−/−</sup> PMN→BLT1<sup>−/−</sup>) at 20 h and 4 d. (d) Representative flow cytometric analysis of Gr-1<sup>+</sup> neutrophils in the blood and ankle synovial fluid from WT PMN→BLT1<sup>−/−</sup> mice at 20 h and 4 d after initial serum and neutrophil transfer. For these experiments, transferred CD45.1<sup>+</sup> WT neutrophils were used to distinguish from endogenous CD45.2<sup>+</sup> BLT1<sup>−/−</sup> neutrophils. For comparison, synovial fluid analysis from WT mice is depicted in Fig. S2. (e) Percentage of transferred Gr-1<sup>+</sup> WT neutrophils out of total neutrophils in the blood and synovial fluid of WT PMN→BLT1<sup>−/−</sup> mice at 20 h and 4 d. (f) Total numbers of transferred WT and endogenous BLT1<sup>−/−</sup> neutrophils within the synovial fluid of WT PMN→BLT1<sup>−/−</sup> mice at 20 h and 4 d. (g and h) Ankle synovial tissue was harvested, and total RNA was isolated from WT PMN→BLT1<sup>−/−</sup> mice at 3 (g) and 12 (h) d after initial serum and adoptive neutrophil transfer. Chemokine expression was measured by qPCR. RNA from six to eight individual ankles was isolated. Error bars represent SEM. *, <i>P</i> < 0.05; **, <i>P</i> < 0.005 (WT vs. WT PMN→BLT1<sup>−/−</sup> mice).
of neutrophils initiates a unique pathway that generates specific proinflammatory mediators that are required for leukocytes to subsequently migrate into sites of inflammation. Although the precise mechanisms by which this process occurs remain to be elucidated, our findings describe a novel and previously unrecognized important biological role for BLT1 in directing neutrophil recruitment in a non–cell-autonomous manner, revealing yet another facet of the intricate biology of leukocyte trafficking.

MATERIALS AND METHODS

Mice. KRN mice were provided by D. Mathis and C. Benoist (Harvard Medical School, Boston, MA). K/BxN mice were obtained by crossing KRN with NOD/LtJ mice (The Jackson Laboratory). BLT1-deficient mice were generated in our laboratory (4). B6.SJL-PtprcaPep3b/BoyJ (CD45.1+) and C57BL/6 mice were from The Jackson Laboratory or the National Cancer Institute. All experiments were performed according to protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Serum transfer and arthritis scoring. Pool serum from 8-wk-old arthritic K/BxN mice was injected into recipient mice (150 μl serum i.p. on days 0 and 2). Hind ankles were measured with a pocket thickness gage (Mitutoyo America), and the average was calculated. Clinical scoring for each paw was based on the following index: 0, no edema/erythema; 1, localized edema/erythema over one surface of paw; 2, edema/erythema involving the entirety of one surface of paw; 3, edema/erythema involving both surfaces of paw. Scores were added for all four paws for a composite score.

Histological scoring. Dissected ankles were fixed in 10% neutral buffered formalin and demineralized in modified Krishnendu’s solution. Histological scoring of inflammation, cartilage erosion, and bone erosion were ranked as follows: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe (23). To quantify synovial hyperplasia and neutrophil accumulation, total cell number was calculated by counting nuclei and total neutrophil numbers were calculated by counting cells with polymorphonuclear morphology within a 0.1-mm² section of synovium.

RNA isolation. RNA was prepared using a modification of the LiCl/urea technique. Ankles synovial cavity contents were lavaged with PBS. Synovial surfaces were immersed in 6 M urea/2% SDS. The RNA solution was precipitated with 6 M urea/6M LiCl/20 mM NaOAc, resuspended with 6 M urea/3M LiCl/20 mM NaOAc, proteinase K treated, and phenol-chloroform extracted.

qPCR. Total RNA was DNasel (Invitrogen) treated. 10–50 ng total RNA was used as template for the RT reaction. 50 μl cDNA was synthesized using oligo(dT)₁₅, random hexamers, and multiscribe reverse transcriptase (Applied Biosystems). All samples were reverse transcribed under the same conditions and from the same RT master mix to minimize differences in RT efficiency. The qPCR reaction was performed with 5 μl cDNA, 12.5 μl 2X SYBR green master mix (Stratagene), and 250 nmol sense and antisense primer. All oligonucleotide primers for qPCR (Table S1, available at http://www.jem.org/cgi/content/full/jem.20052349/DC1) were from Invitrogen. Emitted fluorescence for each reaction was measured three times during the annealing/extension phase, and amplification plots were analyzed using MX4000 software version 3.0 (Stratagene). Quantitative values for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities, and the calculated number of copies was divided by the number of copies of the housekeeping gene β₂ microglobulin.

Administration of CP-105,696. CP-105,696 was provided by Pfizer. 10 mg/kg CP-105,696 was administered via gavage in 10% EtOH, 0.5% methylcellulose, and 0.5% Tween 80.

Bone marrow neutrophil adoptive transfer. Marrow cavities of the tibias and femurs of 8-wk-old donor mice were flushed with DMEM 10% FCS media. After RBC hypotonic lysis, mature neutrophils were isolated by centrifugation over discontinuous Percoll gradients at 500 g for 30 min at 28°C, consisting of 55 (vol/vol), 65 (vol/vol), and 75% (vol/vol) Percoll in PBS. Mature neutrophils recovered at the interface of the 65 and 75% fractions were ≥90% pure and ≥95% viable as determined by Wright-Giemsa staining and trypan blue exclusion, respectively. 5 × 10⁶ neutrophils in HBSS were injected i.v. into 8-wk-old recipient mice. For the neutrophil transfer experiments, 150 μl K/BxN serum was injected i.v. on days 0 and 2.

Flow cytometry. Ankle joint synovial fluid was lavaged with 5 μl HBSS/5% FCS, and contents from both ankles were pooled for each mouse. Synovial fluid leukocytes were counted using a hemacytometer. Leukocytes were incubated with 2.4G2 anti-FcγII/III receptor and stained with PE-conjugated anti–mouse CD45.1, FITC-conjugated anti-mouse CD45.2, and APC-conjugated anti–mouse Gr-1 (BD Biosciences). Cytofluorometry was performed with a FACScalibur Cytometer (Becton Dickinson). Neutrophils were identified by characteristic forward/side scatter and Gr-1 positivity. Results were analyzed with Flojo software (Tree Star).

Immunofluorescent staining. Ankles harvested 7 d after serum transfer were cut in 5-μm frozen sections on a cryomicrotome support using a tape transfer method. After blocking, slides were stained with an Fc-specific FITC-labeled F(ab')₂ fragment of goat anti–mouse IgG (Jackson Immunoresearch Laboratories). Tissue sections were visualized using a fluorescent microscope, and digital photographs were processed using Adobe Photoshop (Adobe Systems).

Statistical analysis. Comparisons were analyzed for statistical significance by two-tailed Student’s t test using Microsoft Excel software, with P < 0.05 considered significant.

Online supplemental material. Fig. S1 depicts representative flow cytometry of the bone marrow neutrophil population used for neutrophil adoptive transfer experiments. Fig. S2 depicts representative flow cytometry of ankle synovial fluid from WT mice 20 h and 4 d after serum transfer. Table S1 lists primers used for qPCR. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052349/DC1.

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