Neutrophil-derived leukotriene B_4 is required for inflammatory arthritis

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Neutrophils serve as a vanguard of the acute innate immune response to invading pathogens. Neutrophils are also abundant at sites of autoimmune inflammation, such as the rheumatoid joint, although their pathophysiologic role is incompletely defined and relevant effector functions remain obscure. Using genetic and pharmacologic approaches in the K/BxN serum transfer model of arthritis, we find that autoantibody-driven erosive synovitis is critically reliant on the generation of leukotrienes, and more specifically on leukotriene B_4 (LTB_4), for disease induction as well as perpetuation. Pursuing the cellular source for this mediator, we find via reconstitution experiments that mast cells are a dispensable source of leukotrienes, whereas arthritis susceptibility can be restored to leukotriene-deficient mice by intravenous administration of wild-type neutrophils. These experiments demonstrate a nonredundant role for LTB_4 in inflammatory arthritis and define a neutrophil mediator involved in orchestrating the synovial eruption.

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The clinical hallmarks of rheumatoid arthritis, a prevalent disease that affects 1% of the population, include polyarticular joint inflammation with leukocytic recruitment into synovial fluid and tissue, hyperplasia of the synovial joint lining, and development of synovial pannus that is erosive into cartilage and bone. Mechanistically, strong evidence implicates autoreactive lymphocytes and antibodies in disease pathogenesis, yet the effector mechanisms recruited to engender synovial inflammation remain obscure. From a cellular standpoint, the rheumatoid arthritis synovium is variably populated with numerous leukocytic lineages; lymphocytes, plasma cells, macrophages, neutrophils, and mast cells are all present. Furthermore, the inflammatory synovial fluid contains dramatically elevated numbers of leukocytes comprised predominantly of neutrophils (1–3). Functionally, although macrophages appear to provide a substantial source of proinflammatory cytokines, the contribution of other leukocyte populations to synovial inflammation remains largely speculative. In addition to cytokines, the leukotrienes are among the inflammatory mediators expressed in the inflamed joint. Indeed, previous analyses document marked elevation of both leukotriene B_4 (LTB_4) and the cysteinyl leukotrienes in diseased joints (2, 4).

The murine K/BxN serum transfer model has provided insight into the pathogenic mechanisms contributing to the effector phase of autoimmune synovitis. Distal symmetric erosive polyarthritis in K/BxN transgenic mice proceeds from pathogenic autoantibodies generated from interaction between T and B lymphocytes via the MHC class II molecule A^{g7}. Autoimmune interactions in the adaptive immune system thus constitute proximal pathogenic events in disease development. The effector phases of this autoantibody-mediated arthritis, which can evolve in the absence of lymphocytes, can be induced by passive transfer of IgG containing serum to recipient mice (5). Essential effector phase mechanisms elucidated thus far include the complement anaphylatoxin C5a, FcγRIII, TNF, and IL-1 receptor 1 (including by inference, IL-1; references 6 and 7). From a cellular standpoint, mast cells and neutrophils are essential, with an additional role for NK-T cells and down-modulating activity demonstrated for macrophages (8–12). These insights notwithstanding, the proinflammatory mediators contributed by these lineages remain elusive. Herein,

The online version of this article contains supplemental material.

we demonstrate a critical contribution of neutrophil-derived LTB_4 to arthritis induction and perpetuation in the K/BxN serum transfer model of inflammatory arthritis.

RESULTS AND DISCUSSION

Leukotrienes are present in arthritic joints

To determine whether arthritic K/BxN joint tissues demonstrate elevated LTB_4 levels and thereby mimic human arthritis pathophysiology, we assayed leukotriene concentrations in joint tissues from arthritic and control nonarthritic mice. As shown in Fig. 1 (A and B), significantly elevated LTB_4 and cysteinyl leukotriene C_4 (LTC_4) levels (161 ± 20 pg LTB_4 and 139 ± 9 pg LTC_4 per gram ankle tissue) were detected in joint tissues from K/BxN mice with chronic arthritis. No leukotrienes were reproducibly detected in joint tissue from nonarthritic C57BL/6 mice. To assess a temporal relationship between the generation of leukotrienes and the development of clinical arthritis, we performed a time-course analysis of joint LTB_4 levels after the administration of arthritogenic K/BxN serum. Indeed, ankle tissues show increasing concentrations of LTB_4 that correlate strongly with increasing arthritis severity through disease establishment (Fig. 1 C).

Leukotriene deficient mice are resistant to arthritis

Elevated tissue levels of leukotrienes in arthritic mice prompted genetic exploration of a role for leukotrienes in K/BxN serum transfer arthritis. Because leukotriene synthesis proceeds via conversion of arachidonic acid to leukotriene A_4 (LTA_4) through coordinate enzymatic modification by the enzyme 5-lipoxygenase (5-LO) and the 5-LO activating protein, we assessed arthritic responses to arthritogenic K/BxN serum in mice deficient in the 5-LO enzyme. In contrast to WT control mice, 5-LO null mice are remarkably resistant to development of K/BxN serum–induced inflammatory arthritis (Fig. 2 A). Inflammation, bone erosion, and cartilage erosion assessed histomorphometrically confirm clinical findings in 5-LO null mice. Whereas WT mice demonstrate synovial hyperplasia, leukocytic infiltration, and presence of synovial erosion into bone and cartilage, 5-LO null joint tissues demonstrate a normal appearance, with little evidence of these inflammatory changes (Fig. 2, D and G).

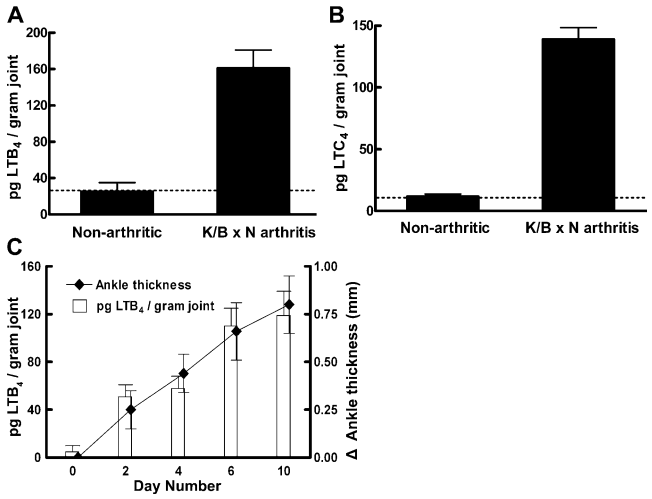

Figure 1. Quantification of leukotrienes in arthritic joint tissues. LTB_4 (A) or LTC_4 (B) was quantified in ankle tissues from arthritic K/BxN mice ($n = 5$) or control B6 mice ($n = 10$). Values depicted are means ± SEM of three independent experiments ($P < 0.001$). Dotted line represents lower limit of assay sensitivity. (C) Kinetics of LTB_4 production in arthritic joint tissue. B6 mice were administered arthritogenic K/BxN serum and killed at 2-d increments thereafter for quantification of ankle tissue LTB_4 (bars). Clinical arthritis was measured throughout the experimental course (◆). Data represent means ± SEM of five mice per time point in three separate experiments.

Having identified a critical requirement for leukotrienes in K/BxN serum transfer arthritis, we sought to define the leukotriene species mediating this pathogenic event. Subsequent to synthesis of the unstable intermediate LTA_4, further metabolism to potently bioactive leukotriene metabolites can proceed either by conjugation to glutathione to form the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) or hydrolysis to form LTB_4. The enzyme leukotriene C_4 synthase (LTC_4S) catalyzes the conjugation of glutathione to LTA_4 to form LTC_4 and thus is required for the generation of all cysteinyl leukotrienes. Potent bioactivities of the cysteinyl leukotrienes relevant in inflammatory arthritis include induction of vasodilation and increased vascular permeability, as well as stimulation of cytokine secretion from mast cells (13, 14). To assess a role for cysteinyl leukotrienes in induction of K/BxN serum transfer inflammatory arthritis, we examined the arthritic responses in LTC_4S null mice. Interestingly, we find that these mice demonstrate robust clinical evidence of arthritis (Fig. 2 B). Histomorphometric scoring of inflammation, bone erosion, and cartilage erosion confirms clinical findings with prominent leukocytic infiltration and synovial hyperplasia in both WT and LTC_4S null mice (Fig. 2, E and H). Thus, synovial inflammation may proceed in the absence of the cysteinyl leukotrienes.

LTA_4 hydrolase (LTA_4H) catalyzes the hydrolysis of the unstable epoxide intermediate LTA_4 to LTB_4. Among the bioactivities attributable to LTB_4 are increased vascular permeability, potent leukocyte chemoattraction, induction of vascular adhesion molecule expression, and stimulation of neutrophil degranulation (15). Thus, LTB_4 was a prominent candidate effector molecule for participation in this neutrophil-dependent arthritis model. Indeed, LTA_4H null mice are remarkably resistant to K/BxN serum transfer arthritis (Fig. 2 C). Histomorphometric analyses concur with clinical observations (Fig. 2, F and I), as LTA_4H null mice demonstrate little evidence of the leukocytic infiltration or synovial hyperplasia apparent in WT mice. Thus, these data demonstrate that the requirement for leukotrienes in this model is a requirement for LTB_4.

Treatment of arthritis via leukotriene inhibition

To independently confirm a critical role for leukotrienes in arthritis induction after K/BxN serum transfer, we used a pharmacologic inhibitor of 5-LO. Mice were orally administered

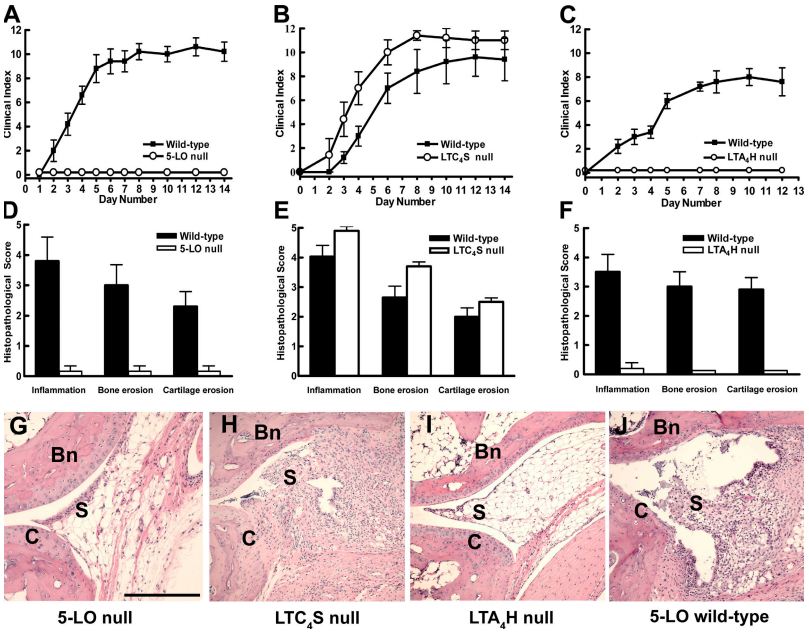

Figure 2. LTB_4–deficient mice are resistant to arthritis. Arthritis responses in 5-LO null (A), LTC_4S null (B), LTA_4H null (C), and control mice. $n = 5$ mice/group/experiment, representative of three experiments. 5-LO null and LTA_4H null versus WT, $P < 0.001$. (D–F) Histomorphometric arthritis quantification from 5-LO null (D), LTC_4S null (E), and LTA_4H null (F) mice. $P < 0.001$, 5-LO null and LTA_4H null. (G–J) Histologic sections from 5-LO null (G), LTC_4S null (H), LTA_4H null (I), and WT (J) mice at experimental day 14. Bar, 100 μm. S, synovium; C, cartilage; Bn, bone.

either 5-LO inhibitor (L-739,010; 250 mg/kg, twice daily) or carrier control beginning 2 d before K/BxN serum injection (16). 5-LO activity was assessed at pharmacologic peak and trough on peripheral blood leukocytes to confirm that blockade of leukotriene synthesis was achieved with this regimen (see Supplemental Materials and methods and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052371/DC1). Consistent with our genetic observations, oral administration of the 5-LO inhibitor effectively prevents induction of arthritis (Fig. 3, A and B). Histologically, 5-LO inhibitor–pretreated mice display little evidence of leukocytic infiltrate, synovial hyperplasia, or joint erosions, whereas control mice display marked arthritic activity (Fig. 3 C).

Mechanistically, genetic approaches using knockout mice are limited to defining a role for leukotrienes in disease induction. An ongoing role in disease perpetuation remained undefined. We therefore used the 5-LO pharmacologic inhibitor to define an ongoing role for leukotriene synthesis after establishment of K/BxN serum transfer arthritis. Here, mice were administered K/BxN serum and allowed to develop robust clinical evidence of arthritis. These arthritic mice were thereafter administered either 5-LO inhibitor or vehicle control and monitored for clinical arthritis activity. We find that administration of the 5-LO inhibitor decreases clinical arthritis to ∼20% of that evident in control vehicle–treated mice at 14 d after arthritis induction (Fig. 3, A and B). Histologic examination reveals decreased tissue inflammation, synovial hyperplasia, and erosive activity (Fig. 3 C). Focusing on the striking decline in leukocytic infiltration in synovial tissues of 5-LO inhibitor–treated mice, we quantified synovial tissue neutrophils in treated and control mice (Fig. 3 D). We observe a profound decline in synovial neutrophil numbers to 26% of control mice after inhibition of 5-LO. This decrease in neutrophil numbers accounts for 87% of the total decrease in synovial cellularity (not depicted), suggesting that neutrophil recruitment comprises a prominent activity of LTB_4 in the established phase of K/BxN arthritis. This assertion is supported by the ongoing requirement for the LTB_4 receptor, BLT1, on neutrophils in established arthritis and by the therapeutic efficacy of a BLT1 antagonist for ameliorating K/BxN arthritis (17).

Leukocytes contribute leukotriene synthetic function

We next sought to define cellular sources of leukotriene synthesis in inflamed synovium. 5-LO expression is limited predominantly to leukocyte populations; however, there are reports of 5-LO activity in nonhematopoietic lineages. To focus our analyses, we used radiation chimeric mice and found that arthritogenic 5-LO synthetic activity derives exclusively from a BM lineage (Fig. 4 A and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20052371/DC1). Because analyses of BM lineage–specific studies demonstrate critical requirements for mast cells and neutrophils in K/BxN arthritis, we specifically explored the contribution of leukotriene synthetic function from these lineages. To assess a requirement for synovial mast cell 5-LO activity, we used a modification of the genetic approach used previously to demonstrate that synovial mast cells contribute to K/BxN arthritis pathogenesis in mast cell–deficient W/W_v mice

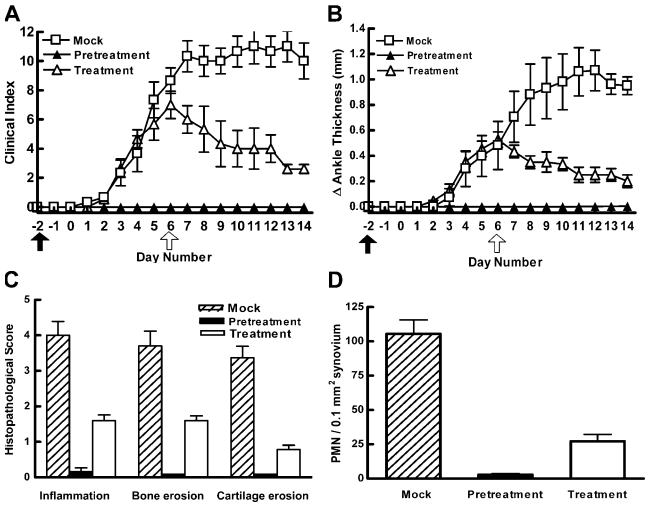

Figure 3. 5-LO inhibitor (L-739,010) ameliorates arthritis. (A and B) L-739,010 administered either as pretreatment (filled arrow) or as a therapeutic regimen (open arrow). $n = 5$ mice/group/experiment, representative of three experiments. $P < 0.001$ pretreatment versus mock-treated mice; $P < 0.01$ treatment versus vehicle. (C) Histomorphometric quantification of arthritis at experimental day 14. $P < 0.01$–0.001. (D) Quantification of neutrophil accumulation per 0.1-mm^2 synovial area. $n = 5$ mice/group/experiment, 20–25 fields/mouse, representative of three experiments. $P < 0.01$, pretreated and treated versus mock treatment.

(8). Interestingly, 5-LO null mast cell–engrafted W/W_v mice demonstrate the same degree of arthritic response as W/W_v mice engrafted with WT mast cells (Fig. 4 B). Thus, mast cells are not a critical cellular source of arthritogenic LTB_4.

Neutrophils contribute to arthritis via LTB_4 production

To define a functional role for neutrophil leukotriene production in K/BxN serum transfer arthritis induction, we adoptively transferred WT neutrophils into 5-LO null recipients. Indeed, we find that neutrophils can functionally complement leukotriene deficiency, restoring ~83% of arthritic activity (Fig. 4 C and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20052371/DC1). Confirming that the relevant role of neutrophils in this system is to provide LTB_4, we found that coadministration of the 5-LO inhibitor completely inhibits arthritic activity from adoptively transferred neutrophils, the only source of 5-LO activity in recipient mice (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20052371/DC1). To establish that WT donor neutrophils may contribute locally to synovitis, we transferred WT congenic CD45.1 donor neutrophils into 5-LO null mice (CD45.2 background). Indeed, immunofluorescence analysis of arthritic recipient 5-LO–deficient synovial tissues reveals abundant WT donor neutrophils as well as recipient neutrophils in the disease lesion (Fig. 4 H).

In addition to defining a critical, ongoing requirement for LTB_4 in the effector phase of synovial inflammation in the K/BxN model, these findings establish a novel mechanistic contribution of neutrophils to autoimmune disease. Typically, neutrophils are envisaged as a responding lineage, recruited via chemoattractants such as LTB_4 to provide pathogen-destroying effector functions as part of an innate immune response. Here, we demonstrate that neutrophils contribute essentially to the establishment of the autoimmune inflammatory lesion via synthesis of LTB_4, inciting disease through the potent bioactivities of this lipid inflammatory mediator.

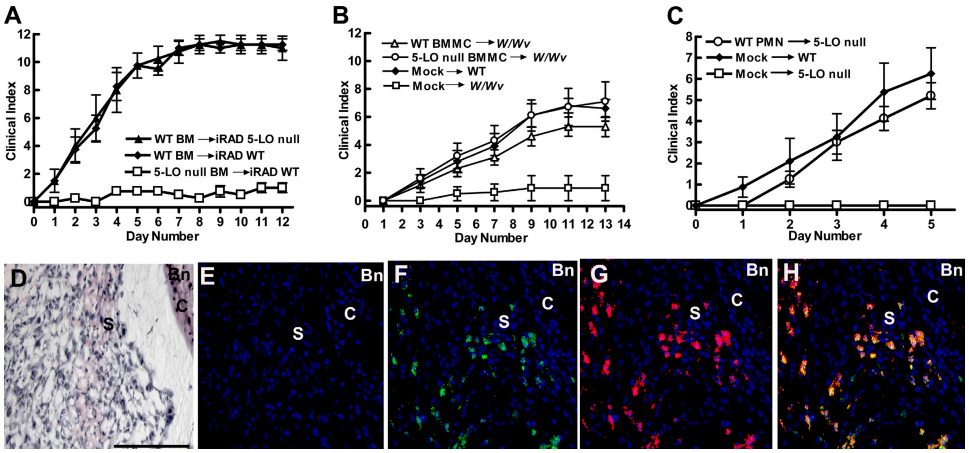

Figure 4. Neutrophils are a cellular source of synovial arthritogenic leukotrienes. (A) 5-LO activity derived from BM lineages. Lethally irradiated (iRAD) recipient mice were reconstituted with donor BM as labeled. $n = 5$ mice/group/experiment, representative of three experiments. $P < 0.001$. (B) Mast cell 5-LO activity is dispensable for arthritis induction. Mast cell–deficient W/W^v mice were engrafted as labeled and assessed for arthritic response. $n = 10$–12 mice/group, $P < 0.001$, WT and 5-LO null BM-derived mast cell versus W/W^v mice. (C) Neutrophils provide arthritogenic LTB_4. Purified neutrophils from WT mice were transferred daily for 5 d into 5-LO null recipient mice. $n = 5$ mice/group/experiment, representative of three experiments. $P < 0.05$. (D–H) WT donor neutrophils (CD45.1) in recipient 5-LO null joint tissues (CD45.2). Shown are: (D) hematoxylin/eosin stain, (E) isotype control, (F) CD45.1 (green), (G) Gr-1 (red), and (H) merge CD45.1 and Gr-1 with nuclear strain (blue). Bar, 25 μm.

These findings confirm and extend previous observations regarding the role of leukotrienes (18–20) and neutrophils in animal models of arthritis and may provide insight into human inflammatory disease. In inflammatory arthritis, the articular cavity can become dramatically infiltrated with neutrophils, with a turnover estimated at a billion cells per day in a single joint (1–3). Yet their role in pathogenesis is incompletely understood. Are they simply responding to chemoattractants generated by other (e.g., autoreactive) cells, or do they have a more primary role in promoting inflammation within the confines of the joint? Although both roles may in fact contribute to disease, our findings lend mechanistic support to the possibility that neutrophils within the joint may participate directly in inflammation via elaboration of LTB_4. This possibility is consistent with the marked elevation of LTB_4 in neutrophil-rich synovial effusions (2). Because the notion of neutrophils as instigators of disease has not been extensively explored, in part because of the technical limitations of working with this lineage, these lessons have broad import for human autoimmune disease. Further attention to neutrophils promises surprising insights into their contributions to diverse autoimmune conditions, aiding identification of novel direct targets of therapy.

MATERIALS AND METHODS

Mice. 6–10-wk-old mice were used for these studies. C57BL/6J, B6.SJL-Ptprc^a Pep3^b/BoyJ (CD45.1 allele), and WBB6F1-*W/W$_v$* mice were purchased from The Jackson Laboratory, and 129SvEv mice were purchased from Taconic. 5-LO null mice (N10 backcross to B6; reference 21), LTA_4H null mice (129SvEv background; provided by B.H. Koller, University of North Carolina Chapel Hill, Chapel Hill, NC; reference 22), and LTC_4S null mice (N5 backcross to B6; reference 23) were bred locally. K/BxN mice were maintained as described previously (5). All procedures were approved by the Dana-Farber Cancer Institute Institutional Animal Care and Use Committee.

Arthritis studies. Arthritogenic K/BxN serum was transferred to recipient mice, and clinical arthritis responses were graded as described previously (5, 8, 24).

Measurement of tissue leukotrienes. Leukotriene levels in joint tissue homogenates were quantified using HPLC separation followed by ELISA measurement as described previously (23).

Pharmacologic inhibition of 5-LO. A 5-LO inhibitor, L-739,010 (2-cyano-4-(3-furyl)-7-[[6-[3-(3-hydroxy-6,8-dioxabicyclo [3.2.1] octanyl)]2-pyridyl] methoxy]naphthalene; provided by Merck Frosst Centre for Therapeutic Research; reference 16) was dissolved in 1% methylcellulose in PBS (Sigma-Aldrich) and administered orally via gavage twice daily. The dose used, 250 mg/kg, was chosen based on the previously defined pharmacokinetic profile of L-739,010 in mice. A vehicle control (1% methylcellulose) was administered orally in the same volume and frequency to control mice.

Generation of radiation BM chimeras. Recipient mice were lethally irradiated with split doses (500 and 450 cGy), transplanted with donor BM, and supported with oral antibiotic (Baytril). Arthritis experiments were performed after allowing 8 wk for transplant engraftment.

BM-derived mast cell cultures and mast cell engraftment. BM-derived mast cells were generated and engrafted into mast cell–deficient *W/W^v* recipients as described previously (8).

BM neutrophil isolation and engraftment. Isolation of mature neutrophils from BM was accomplished using discontinuous Percoll density centrifugation (25). Mature neutrophils were recovered at the interface of the 65 and 75% fractions. Neutrophil purity (>90%) was determined both morphometrically by Diff-Quik staining and cytofluorometric expression of Gr-1 (Fig. S3). 5-LO null recipient mice were administered 10^7 neutrophils via tail vein injection on days 0, 1, 2, 3, and 4 after K/B x N serum transfer.

Histological examination and quantification of neutrophil accumulation of murine synovial tissues. Arthritis changes in joint tissues were graded based on the scoring system used by Pettit et al. (26) with minor modifications. Specifically, cartilage was scored using the following criteria: 0, no cartilage injury; 1, synovial adherence to margins of cartilage in fewer than three sites; 2, synovial adherence to margins of cartilage in three or more sites; 3, synovial adherence to cartilage not limited to margins but no full-thickness injury (damage does not extend beyond tidemark); 4, full-thickness injury in fewer than three sites; 5, full-thickness injury in three or more sites. To quantify synovial neutrophil accumulation of murine synovial tissues, total neutrophil numbers were calculated by counting cells with polymorphonuclei morphology in 0.1-mm^2 sections of synovium. A minimum of eight sections per mouse and five reticule-defined regions (0.1 mm^2) per section were assessed. Immunofluorescence histology of joint tissues was performed as described previously (24) with the following directly conjugated mAbs: FITC anti-CD45.1 antibody (clone A20; SouthernBiotech), FITC anti-IgG2a antibody (SouthernBiotech), PE anti–Gr-1 antibody (clone RB6-8C5; Caltag Laboratories), and PE anti-IgG2b antibody (Caltag Laboratories). Tissue sections were examined under a Nikon TE2000-U inverted fluorescence microscope equipped with a high resolution Sony ICX285 Cooled CCD digital camera.

Statistical analysis. Data are presented as the mean ± SEM. Statistical significance for comparisons between groups was determined using Student's paired two-sample *t* test or ANOVA followed by Bonferroni correction.

Online supplemental material. Fig. S1 confirms the inhibition of 5-LO activity via oral administration of L-739,010 through ex vivo assay of whole blood leukocytes. Fig. S2 demonstrates the degree of BM engraftment in radiation chimeric mice. Fig. S3 shows neutrophil purification for adoptive transfer. Fig. S4 verifies that adoptively transferred neutrophils supply 5-LO activity for arthritis restoration. Online supplemental material, including Supplemental Materials and methods, is available at http://www.jem.org/cgi/content/full/jem.20052371/DC1.

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