Lipid-Cytokine-Chemokine Cascade Drives Neutrophil Recruitment in a Murine Model of Inflammatory Arthritis

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SUMMARY

A large and diverse array of chemoattractants control leukocyte trafficking, but how these apparently redundant signals collaborate in vivo is still largely unknown. We previously demonstrated an absolute requirement for the lipid chemoattractant leukotriene B4 (LTB4) and its receptor BLT1 for neutrophil recruitment into the joint in autoantibody-induced arthritis. We now demonstrate that BLT1 is required for neutrophils to deliver IL-1 into the joint to initiate arthritis. IL-1-expressing neutrophils amplify arthritis through the production of neutrophil-active chemokines from synovial tissue cells. CCR1 and CXCR2, two neutrophil chemokine receptors, operate nonredundantly to sequentially control the later phase of neutrophil recruitment into the joint and mediate all neutrophil chemokine activity in the model. Thus, we have uncovered a complex sequential relationship involving unique contributions from the lipid mediator LTB4, the cytokine IL-1, and CCR1 and CXCR2 chemokine ligands that are all absolutely required for effective neutrophil recruitment into the joint.

INTRODUCTION

Leukocyte recruitment into tissue compartments is a tightly regulated process central to all inflammatory responses (Luster et al., 2005). This process is choreographed by a diverse array of chemoattractants, including lipid metabolites (e.g., leukotrienes), proteolytic fragments of serum proteins (e.g., complement), and secreted chemotactic cytokines (e.g., chemokines). These chemoattractants cooperate to control leukocyte migration by activating a family of related chemoattractant G protein-coupled receptors (GPCRs) (Shimizu, 2009; Viola and Luster, 2008). Although chemoattractants appear to have overlapping functions in vitro, in vivo studies have uncovered a complexity in the control of leukocyte migration not appreciated by in vitro chemotaxis assays. This undoubtedly reflects the different temporal and spatial nature of chemoattractant production and activity, inherent differences in the biophysical properties of the different chemoattractants, and ligand-induced regulation of chemoattractant receptor expression and function in vivo.

The K/BxN model of inflammatory arthritis is an excellent model in which to interrogate the complexities of the chemoattractant receptor system in the fine control of leukocyte trafficking in vivo. This murine model of arthritis bears important clinical and histopathologic similarities to human rheumatoid arthritis, with synovial pannus formation and bone cartilage erosions (Kouskoff et al., 1996). K/BxN mice produce antibodies against glucose 6-phosphate isomerase (GPI), which selectively deposit in the joints as immune complexes and fix complement (Matsumoto et al., 2002). After receiving serum from K/BxN transgenic mice containing arthritogenic anti-GPI, wild-type (WT) mice develop an inflammatory arthritis clinically and pathologically similar to the transgenic mice. The innate immune system is required for inflammation in the serum transfer model. Neutrophils, Fcγ receptors, IL-1, the adaptor protein MyD88, the alternative complement pathway, and the C5a receptor C5aR are all required for the generation of arthritis, but T and B lymphocytes are dispensable (Ji et al., 2002a, 2002b; Wipke and Allen, 2001).

We and others have recently contributed to the appreciation of the complexity of chemoattractant control of leukocyte trafficking in this model by discovering that the lipid chemoattractant leukotriene B4 (LTB4) and its high-affinity receptor BLT1 are absolutely required for the development of arthritis in the K/BxN serum transfer model (Chen et al., 2006; Kim et al., 2006). Although we found that neutrophils are required to both express BLT1 and synthesize its ligand, LTB4, to sustain joint inflammation, adoptive transfer of WT neutrophils into arthritis-resistant BLT1 gene-deficient Ltb4r1−/− mice restored arthritis by inducing endogenous Ltb4r1−/− neutrophil recruitment into the joint. This indicated that BLT1-expressing neutrophils enable other neutrophils to enter the joint via chemoattractant receptors other than BLT1.

We have been developing the concept that diverse chemoattractants, including lipids and chemokines, cooperate to control leukocyte recruitment in vivo (Luster and Tager, 2004;...
Medoff et al., 2008). For example, we have found that the LTB4-BLT1 pathway recruits the first wave of antigen-specific T helper 2 (Th2) cells into the lung in sensitized mice after aerosolized antigen challenge (Tager et al., 2003). These Th2 cells then amplify Th2 cell recruitment through the induction of IL-4-inducible chemokine production from a resident lung cell (Medoff et al., 2009). In the current study, we have explored the hypothesis that in the K/BxN model, the LTB4-BLT1 pathway delivers neutrophils expressing IL-1 and chemokines into the joint, which in turn amplifies and sustains neutrophil recruitment and inflammation via both neutrophil-derived and IL-1-inducible chemokines.

RESULTS

Exogenous IL-1β Restores Arthritis Susceptibility to Ltb4r1−/− Mice

Because IL-1 is known to be required for arthritis in the K/BxN model (Ji et al., 2002b), and we previously demonstrated that IL-1 was markedly decreased in the joints of arthritis-resistant Ltb4r1−/− mice (Kim et al., 2006), we first tested whether exogenous murine recombinant IL-1β (mIL-1β) could restore arthritis in Ltb4r1−/− mice. Injection of mIL-1β in addition to K/BxN serum induced polyarthritis in Ltb4r1−/− mice similar to age-matched C57BL/6 wild-type (WT) control mice that received K/BxN serum (Figures 1A and 1B). Although the disease course in Ltb4r1−/− mice that received mIL-1β (mIL-1β → Ltb4r1−/−) was almost identical to WT mice in the early phase, arthritis resolved more quickly in Ltb4r1−/− mice after the last dose of IL-1β.

Histological analysis corroborated our clinical findings (Figure 1C). The joints of WT arthritic mice demonstrated characteristic inflammation with leukocytic infiltrates, synovial hypertrophy, and joint erosion, which was absent or only minimally present in Ltb4r1−/− mice. However, after receiving mIL-1β, Ltb4r1−/− mice developed pathological changes similar to those observed in WT mice, particularly early in disease. Histological scoring confirmed similar inflammation and cartilage and bone erosion in the synovium of WT controls and mIL-1β → Ltb4r1−/− mice in the early stages of arthritis (Figure 1D). By day 12, the leukocytic infiltrates in the synovium of mIL-1β → Ltb4r1−/− mice were significantly reduced. Thus, exogenous mIL-1β was able to bypass the requirement for BLT1 signaling, at least transiently, to induce arthritis in Ltb4r1−/− mice.

To determine whether mIL-1β induced chemokine expression in the synovium of Ltb4r1−/− mice, resulting in the recruitment of Ltb4r1−/− polymorphonuclear leukocytes or neutrophils (PMNs) into the joint in a BLT1-independent manner, we examined the expression of neutrophil-active chemokines in the three groups of mice—WT, Ltb4r1−/−, and mIL-1β → Ltb4r1−/−—on day 4 after the injection of arthritogenic serum (Figure 1E). As expected, Ltb4r1−/− mice showed a markedly attenuated induction of chemokines, IL-1β, and TNF-α compared to WT mice. In mIL-1β → Ltb4r1−/− mice, the levels of several neutrophil-active chemokines, including CCL4 (MIP-1β), CXCL2 (MIP-2), and CXCL1 (KC), were significantly upregulated compared to Ltb4r1−/− mice that did not receive mIL-1β. Notably, IL-1β and TNF-α were not upregulated in mIL-1β → Ltb4r1−/− mice compared to Ltb4r1−/− mice. Therefore, our findings suggest that IL-1β acts downstream of BLT1 signaling to induce chemokine production in synovial tissues during inflammatory arthritis.

To further analyze the importance of chemokines in the recruitment of leukocytes into the joint, synovial fluid leukocytes were isolated from the ankle joints and analyzed for chemokine receptor expression. WT mice had greater amounts of all chemokine receptors tested (CCR1, CCR2, CCR5, CXCR2, CXCR4, BLT1), whereas Ltb4r1−/− mice showed significantly lower amounts, indicating a lack of leukocyte infiltrate into the joints of these mice. After injection of mIL-1β, there was a restoration of all chemokine receptors in joint fluid leukocytes in Ltb4r1−/− mice, consistent with the restoration of leukocyte recruitment into the joints. In particular, high amounts of CCR1 and CXCR2 were observed. Thus, mIL-1β functions to bypass BLT1 and induce the downstream expression of neutrophil-active chemokines, which further recruit PMNs via their specific chemokine receptors.

Neutrophil-Derived IL-1 Is Sufficient to Restore Arthritis Susceptibility in Ltb4r1−/− Mice

We previously demonstrated that WT PMNs were able to restore arthritis in Ltb4r1−/− mice (Kim et al., 2006). Having demonstrated that exogenous IL-1β also restored arthritis in Ltb4r1−/− mice (Figure 1), we next tested whether PMNs were the source of IL-1β. To determine this, bone marrow (BM) PMNs from either WT or IL-1β-deficient Il1b−/− mice were adoptively transferred into Ltb4r1−/− mice, and WT and Ltb4r1−/− mice without adoptive PMN transfer served as controls. As predicted, Ltb4r1−/− mice that received WT PMNs (WT PMN → Ltb4r1−/−) transiently developed arthritis, with an early disease course that was indistinguishable from WT mice, but their arthritis resolved more quickly. In contrast, Il1a−/−/Il1b−/− PMNs could not restore arthritis in Ltb4r1−/− mice (Il1a−/−/Il1b−/− PMN → Ltb4r1−/−) (Figures 2A and 2B), indicating that neutrophil-derived IL-1 is sufficient to restore disease in Ltb4r1−/− mice.

Histological and chemokine analyses of these mice also paralleled their clinical disease activity. Joints of WT mice and WT PMN → Ltb4r1−/− mice demonstrated characteristic inflammation with leukocytic infiltrates, synovial hypertrophy, and joint erosion, which were absent or only minimally present in Ltb4r1−/− mice. Histological scoring confirmed similar inflammation and cartilage and bone erosion in the synovium of WT controls and mIL-1β → Ltb4r1−/− mice in the early stages of arthritis (Figure 1D). By day 12, the leukocytic infiltrates in the synovium of mIL-1β → Ltb4r1−/− mice were significantly reduced. Thus, exogenous mIL-1β was able to bypass the requirement for BLT1 signaling, at least transiently, to induce arthritis in Ltb4r1−/− mice.

Activated PMNs Produce IL-1β

To demonstrate that murine PMNs produce IL-1β in the course of K/BxN arthritis, synovial fluid (SF) leukocytes were collected from arthritic ankles of 8-week-old K/BxN mice. SF PMNs were subsequently purified by immunomagnetic isolation via the neutrophil-specific antibody Ly6G to a purity of ~95% (Figure S1 available online). Quantitative PCR (qPCR) analysis confirmed high amounts of IL-1β mRNA in SF PMNs (Figure 3A). We next compared IL-1β mRNA in purified SF PMNs with total SF leukocytes (Figure 3B) from the joints of WT mice on days 1, 3, 7,
**Figure 1.** Exogenous IL-1β Bypasses BLT1 to Induce Arthritis in Ltb4r1<sup>-/-</sup> Mice

(A and B) Clinical score. The development of arthritis was examined in four different groups of mice (WT, Ltb4r1<sup>-/-</sup>, mIL-1β → Ltb4r1<sup>-/-</sup>, and CP [carrier protein] → Ltb4r1<sup>-/-</sup>) after K/BxN serum transfer on days 0 and 2. The mIL-1β → Ltb4r1<sup>-/-</sup> mice were injected with murine recombinant IL-1β i.p. on days 0, 1, and 2, and the CP → Ltb4r1<sup>-/-</sup> mice were injected with the carrier protein BSA on the same days. Ankle thickness (A) and clinical score (B) of these mice were assessed daily. n = 6–9 mice in each group, and data are representative of three independent experiments. WT versus mIL-1β → Ltb4r1<sup>-/-</sup> p < 0.0001, Ltb4r1<sup>-/-</sup> versus mIL-1β → Ltb4r1<sup>-/-</sup> p < 0.0001 by ANOVA.

(C) Histopathology. In separate experiments, mice from these groups were sacrificed 4, 8, or 12 days after initial serum transfer and ankles harvested, paraformaldehyde-fixed, and H&E stained (scale bar represents 100 μm).

(D) Histological scoring. The degree of inflammatory infiltrates, cartilage erosion, and bone erosion in the ankle sections of these mice was scored according to established methods (Pettit et al., 2001). WT versus mIL-1β → Ltb4r1<sup>-/-</sup> <p < 0.0005; Ltb4r1<sup>-/-</sup> versus mIL-1β → Ltb4r1<sup>-/-</sup> p < 0.005, **p < 0.05.

(E) Synovial tissue chemokine and cytokine levels. Four days after serum transfer, mice were sacrificed and ankle synovial tissue was harvested for chemokine and qPCR analysis (n = 4–10 from two independent experiments).
Lipid-Cytokine-Chemokine Cascade in PMN Homing

We next analyzed the spontaneous release of IL-1β from PMNs. Greater amounts of pro-IL-1β were detected in SF PMNs from K/BxN transgenic mice. This is probably related to a dilution of PMNs with other cells in the bulk synovial fluid, resulting in a dilution of IL-1β protein. It also suggests that PMNs are the main cellular source of IL-1β in the synovial fluid of these mice.

Having established the presence of IL-1β mRNA in SF PMNs, we examined IL-1β protein expression in PMNs. We first measured pro-IL-1β by using immunoblot analysis comparing lysates of three different populations of cells: BM PMNs, SF PMNs, and total SF leukocytes from K/BxN transgenic mice. Greater amounts of pro-IL-1β protein were detected in SF PMNs, although it was not detected in BM PMNs. Consistent with the RNA data, total SF leukocytes contained less IL-1β protein than purified SF PMNs, again probably reflecting a dilution effect. The induction of pro-IL-1β in BM PMNs is probably a result of stimulatory signals received in, or on the way to, the arthritic joint. We next analyzed the spontaneous release of IL-1β by PMNs, determining IL-1β concentrations in the supernatants of purified BM PMNs and SF PMNs from K/BxN transgenic mice incubated ex vivo for 21 hr. In agreement with our immunoblot results, IL-1β was detected in the supernatants of SF PMNs by ELISA, but not in the supernatants of BM PMNs. Finally, we also identified IL-1β protein by ELISA in purified SF PMNs isolated from the joints of WT mice 7 and 10 days after the injection of K/BxN serum. On days 1 and 3 after serum injection, the recovery of synovial fluid neutrophils was too low to detect IL-1β by ELISA.

To identify potential links between LTB₄ and neutrophil production of IL-1β, we also assayed supernatants of BM PMNs and SF PMNs for LTB₄ after 21 hr of ex vivo incubation. Under these conditions, SF PMNs released significantly more LTB₄ compared with BM PMNs. In a separate experiment, SF PMNs were incubated in the presence of the BLT1-specific antagonist CP-105,696, and IL-1β release was assayed. There was no difference in IL-1β release between SF PMNs that were incubated in the presence or absence of CP-105,696. Therefore, endogenously produced LTB₄ had no apparent autocrine or paracrine effect on IL-1β production in SF PMNs ex vivo. We also tested the effect of LTB₄ on IL-1β mRNA and protein release in WT BM PMNs. LTBB₄ also had no effect on IL-1β mRNA or protein amounts in neutrophils, suggesting that LTB₄ is not the stimulus for neutrophil IL-1β production in the K/BxN model.

Because FCγ receptors are essential for the pathogenesis of serum transfer-induced K/BxN arthritis in which immune...
complexes deposit in the joints (Matsumoto et al., 2002), we determined the ability of immune complexes (ICs) to induce IL-1β release from neutrophils. BM-derived PMNs from WT and Ltb4r1/C0/C0 mice and mice deficient in the Fc receptor gamma chain (Fcer1g/C0/C0) were incubated with immobilized ICs. ICs stimulated IL-1β mRNA accumulation in WT and Ltb4r1/C0/C0 BM PMNs but not in Fcer1g/C0/C0 BM PMNs (Figure 3J). Consistent with these RNA data, ICs stimulated IL-1β protein release from WT and Ltb4r1/C0/C0 BM PMNs but not from Fcer1g/C0/C0 BM PMNs (Figure 3K). These data demonstrate that ICs induce IL-1β release from PMNs via FcR activation, as indicated by the fact that IL-1β was not produced by Fcer1g/C0/C0 PMNs. In contrast, Ltb4r1/C0/C0 PMNs produced similar levels of IL-1β mRNA and protein secretion compared with WT PMNs, demonstrating that the LTB4-BLT1 pathway also does not play an indirect autocrine or paracrine role in immune complex-mediated IL-1β production and release by PMNs (Figures 3J and 3K).

Chemokine Induction in Synovial Tissue and Infiltrating Synovial Fluid Leukocytes

We next examined the regulation of chemokine production in the synovia of arthritic WT mice. At different time points (days 1, 3, 7, and 10) after the transfer of arthritogenic serum into WT mice (corresponding to preclinical, early onset, early peak, and established peak disease activities, respectively), synovial tissue was harvested and analyzed for chemokine RNA. We focused on the
ligands for the two main murine neutrophil chemokine receptors CCR1 and CXCR2, because the neutrophil is the main effector cell that drives arthritis in this model. The murine CCR1 ligands include CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), and CCL9 (MIP-1β), and the murine CXCR2 ligands include CXCL1 (KC), CXCL2 (MIP-2), CXCL5 (LIX), and CXCL7 (NAP-2). Synovial tissue chemokine RNA amounts were modulated differently over time (Figure 4A). At day 1, several neutrophil-active chemokines, including the CCR1 ligands CCL4, CCL3, and CCL5 and the CXCR2 ligand CXCL2, were expressed. CCR1 ligands peaked at day 7 whereas CXCR2 ligands continued to increase over time. We also examined chemokine protein in the synovial fluid by ELISA. Consistent with the RNA data, CCR1 and CXCR2 chemokine ligands were detectable by days 1 to 3 and peaked at day 7 with the exception of CXCL2, which continued to rise on day 10.

Chemokine expression in synovial fluid leukocytes was also examined, because leukocytes themselves are a potentially important source of chemokines that amplify inflammatory responses (Figure 4C). Chemokine RNA expression in total bulk leukocytes from joint fluid of inflamed ankles of WT mice was examined at different time points after serum transfer. At day 1, CCL4 was the most highly expressed chemokine in leukocytes recovered from the joint, but it did not increase and was undetectable by day 10. In contrast, CXCL2 increased dramatically starting on day 3 and remained very high through day 10.

To determine whether PMNs themselves also contributed to chemokine production in the joint, PMNs were purified from the synovial fluid and subjected to qPCR analysis (Figure 4D). Interestingly, CXCL2 mRNA was markedly enriched in purified PMNs isolated at all time points, suggesting that PMNs were the main source of leukocyte-derived CXCL2. In contrast, the CCR1 ligand CCL4 was not enriched in the PMN fraction until day 4, suggesting that the earliest source of CCL4 is a leukocyte other than the PMN. Chemokine protein in lysates of purified PMNs from inflamed joints after serum transfer was also analyzed by ELISA. CCR1 and CXCR2 ligands were detected in purified PMNs starting on day 3 when enough PMNs could be recovered for chemokine protein analysis (Figure 4E). Consistent with the RNA data, CXCL2 was the most abundant neutrophil-active chemokine ligand expressed in SF PMNs, although the difference in protein concentration for CXCL2 compared to other chemokines was not as large as the difference in RNA expression.

Immunohistochemical staining of inflamed ankle sections also confirmed chemokine expression at the protein level (Figure 4F). CXCL1 expression was seen primarily in the synovial tissue, whereas CXCL2 was primarily expressed by SF leukocytes. This finding demonstrates that other structural cells of the joint may also contribute to neutrophil recruitment by producing chemokines.

**IL-1β Stimulates Chemokine Production in Endothelial Cells, Fibroblast-like Synoviocytes, and Macrophages**

To determine whether IL-1 delivered to the joint by neutrophils could amplify neutrophil recruitment by inducing the release of neutrophil-active chemokines from synovial tissue cells, we determined the pattern of chemokine production in structural cells of the joint, such as fibroblast-like synoviocytes (FLS), endothelial cells (ECs), and macrophages (MØ), in response to IL-1β and TNF-α stimulation for 4 hr (Figure S2) and 16 hr (Figure 4G). Interestingly, of the CXCR2 ligands, FLS expressed mostly CXCL5 and CXCL1 and not CXCL2 in response to IL-1 stimulation (Figure 4G; Figure S2). Resting and TNF-α-stimulated FLS did not express detectable CXCR2 ligands. Of the CCR1 ligands, only CCL5 was detected in FLS, and it was predominantly induced by TNF-α, rather than IL-1. ECs demonstrated a different pattern of chemokine production. Of the CXCR2 and CCR1 ligands, IL-1β predominantly induced the expression of CXCL1 and CXCL2 at 4 and 16 hr and CCL5 at 16 hr in ECs. MØ had a third pattern of induction. CCL9 and CCL3, CCR1 ligands, and CXCL2, a CXCR2 ligand, were induced by IL-1β and TNF-α, whereas CCL5 was induced by TNF-α. Overall, both IL-1β and TNF-α significantly induced FLS, ECs, and MØ to express neutrophil-active chemokines, with FLS having the highest chemokine RNA expression. Taken together, our findings suggest that neutrophils promote their own recruitment into the joint by releasing large amounts of CXCL2 and by releasing IL-1, which further amplifies neutrophil recruitment by inducing CXCL5 from FLS, CXCL1 from ECs, and CCL9 from MØ.

**Chemokine Receptor Expression on Neutrophils Recruited into the Joint**

To establish the relationship between chemokine expression in the arthritic synovium and the expression of their specific receptors on leukocytes recruited into the joint, we analyzed chemokine receptor expression on joint leukocytes and purified joint neutrophils at different phases of disease after K/BxN serum transfer by qPCR analysis (Figure 5A). On day 1, high CCR1 and CCR5 mRNA expression was detected in the synovial fluid leukocytes. Low amounts of CCR2, CXCR2, and BLT1 were also noted. As disease progressed, CCR5, CXCR2, and CXCR4 expression increased, whereas CCR1 and BLT1 expression remained stable.

Chemokine receptor expression was also evaluated on purified SF PMNs from arthritic joints (Figure 5B). We found that CCR1, CXCR2, and CXCR4 were detectable as early as day 1 after serum transfer and increased throughout the course of arthritis. BLT1 was also expressed early, but by day 7 its expression was not detectable in PMNs.

We also examined BLT1 and CXCR2 protein surface expression by flow cytometry (FCM) analysis with specific mAbs on BM and SF PMNs (Figures 5C–5F). Freshly isolated BM PMNs expressed both BLT1 and CXCR2 on their surface, supporting the notion that these receptors can participate in the recruitment of PMNs into the joint in serum-induced arthritis. However, the patterns of BLT1 and CXCR2 surface expression were different. BLT1 was expressed on all BM PMNs (Figure 5C), whereas CXCR2 was expressed on the surface of a subpopulation of BM PMNs (Figure 5E). Consistent with our RNA data, PMNs isolated from inflamed joints on day 8 after serum transfer had lost their expression of BLT1 (Figure 5D). In contrast, CXCR2 surface expression was now detected on all PMNs recovered from inflamed joints (Figure 5F). In addition, CXCR2 surface expression was also higher than on BM PMNs. Unfortunately, we were unable to investigate CCR1 surface protein expression resulting from the lack of a suitable CCR1 antibody for FCM analysis. We therefore evaluated the expression of CCR1 on BM PMNs.
by qPCR. Consistent with what has been previously reported (Gao et al., 1997), CCR1 was constitutively expressed on murine BM PMNs (Figure S3). These data suggest that during the course of inflammatory arthritis, BLT1 is downregulated on PMNs found in the joint and PMNs expressing CXCR2 are preferentially recruited into the inflamed joint at later time.

Figure 4. Neutrophil-Active Chemokine Expression Follows Temporal and Cell-Specific Patterns in Serum Transfer Arthritis

(A–E) Chemokine RNA in synovial tissue (A), chemokine protein levels in synovial fluid (B), chemokine RNA in synovial fluid leukocytes (C), chemokine RNA (D), and chemokine protein (E) in purified synovial fluid PMNs from the ankles of serum transfer arthritic mice was measured at designated time points during the course of arthritis by qPCR and ELISA (n = 4–7 per time point).

(F) Immunohistochemical staining for CXCL1 and CXCL2 were performed on arthritic ankle tissue sections 7 days after initial serum transfer. Data shown are representative of at least two independent experiments. For each antibody, the left panel scale bars represent 100 μm and the right panel scale bars represent 20 μm.

(G) Production of neutrophil-active chemokines by purified primary mouse fibroblast-like synoviocytes, endothelial cells, and macrophages occurs after 16 hr of stimulation with TNF-α or IL-1β (n = 3–10 from two independent experiments). IL-1β versus TNF-α, *p < 0.05, **p < 0.01, ***p < 0.001; IL-1β versus resting, +p < 0.05, ++p < 0.01, +++p < 0.001.

All data are presented as mean ± SEM.
arthritis. C57BL/6 mice were injected with arthritogenic serum and monitored with clinical measurements (Figure 6C); the joints of Cxcr2+/− mice had a marked delay in the initiation of arthritis compared with WT mice (Figures 6A and 6B), with attenuated arthritis severity. In contrast, arthritis development in the K/BxN serum transfer model, with arthritis mice demonstrated less robust inflammation, synovial hypertrophy, and joint erosion than WT mice, particularly in early time points. In contrast, Cxcr2−/− and WT mice had initially indistinguishable inflammation scores at early points. However, as was seen clinically, the inflammation score in Cxcr2−/− mice improved dramatically by day 12. Interestingly, the bone and cartilage erosion scores did not improve as dramatically as the inflammation score in Cxcr2−/− mice. Taken together these data suggest that CCR1 and CXCR2 both contribute to arthritis development but exert their maximal activity at different time points.

To determine whether CCR1 and CXCR2 account for all of the chemokine receptor activity needed for neutrophil recruitment and arthritis development in the K/BxN serum transfer model, we generated mice genetically deficient in both CCR1 and CXCR2 (Ccr1−/−Cxcr2−/−). Peripheral blood analysis revealed no abnormalities in complete blood count or absolute neutrophil count (Figure S4). However, Ccr1−/−Cxcr2−/− mice were remarkably completely resistant to the development of arthritis (Figures 6A and 6B). Of the Ccr1−/−Cxcr2−/− mice tested (n = 9), only two developed transient joint swelling and erythema that was limited to one portion of the joint and resolved within a few days. Histological scoring showed negligible inflammation and joint destruction (Figures 6C and 6D). These data confirm a critical and nonredundant role for CCR1 and CXCR2 in the recruitment of neutrophils and induction and progression of joint inflammation in this model of arthritis, despite other multiple active chemottractant pathways.

**DISCUSSION**

We have used the K/BxN model of arthritis to study the complex nature of chemotactrant regulation of neutrophil recruitment
into an inflamed joint. In this model, neutrophils are the predominant effector cell and IL-1 is the predominant cytokine that are both required for arthritis. In the present study, we have now linked these two required factors by finding that neutrophils are the main source of IL-1β in this model. Neutrophils isolated from the joints of arthritic mice spontaneously released IL-1β.

Figure 6. CCR1 and CXCR2 Mediate Early and Late Phases of Serum Transfer Arthritis
(A and B) Clinical score. WT, Ccr1−/−, Cxcr2−/−, and Ccr1−/−Cxcr2−/− mice were injected with K/BxN serum on days 0 and 2, and the degree of swelling (A) and clinical score (C) in ankles and forelimbs were measured daily. n ≥ 6 mice per group and are representative of at least two independent experiments. p < 0.0001 between all groups by ANOVA.
(C) Histopathology. In separate experiments, mice from these four groups were sacrificed 4, 8, or 12 days after the first serum transfer, ankles were harvested and paraformaldehyde fixed, and H&E-stained sections were analyzed (scale bar represents 100 μm).
(D) Histopathological scoring. The degrees of inflammatory infiltrates in the processed ankles of these mice were evaluated according to established methods. n ≥ 4 mice per group and are from at least two independent experiments, and statistical comparisons were made between the knockout and WT mice. *p < 0.05, **p < 0.001, ***p < 0.0001.
All data are presented as mean ± SEM.
whereas bone marrow neutrophils from arthritic mice did not, suggesting that factors in the joints of arthritic mice activate neutrophils to synthesize and release IL-1β. We found that ICs, and not LTB₄, induced IL-1β release in BM PMNs in vitro, suggesting that ICs, which are required to initiate arthritis in the model, are the crucial physiological stimulus that induces IL-1β release by PMNs. This is consistent with the findings that FcγR is required for IL-1β production and arthritis development in the model (Ji et al., 2002a).

Mast cells have also been shown to be an important source of IL-1β in the model. The role of mast cells in the model has been controversial, however, because the mast cell-deficient strains WBB6F1-Kit+/Kit+ and WCBB6F1-Kit+/Kit-d have been shown to be resistant to arthritis (Lee et al., 2002), whereas the mast cell-deficient strain C57Bl/6-Kit+/Kit-d has been shown to be completely susceptible to inflammatory arthritis in this model (Zhou et al., 2007). Further studies suggest that these differences may be related to the effect that these mutations have on other cell lineages, such as the neutrophil lineage. For example, Kit+/Kit+ mice have an associated neutropenia, whereas Kit+/- mice have an associated neutrophilia compared to WT mice (Nigrovic et al., 2008). Thus, the mast cell might be an important early source of IL-1β in a relatively neutrophil-deficient environment, whereas the mast cell appears to be dispensable for IL-1β production in a setting where neutrophils are not limiting. This is consistent with the finding that in the mast cell-deficient Kit+/Kit+ strain, a single dose of exogenous IL-1β was able to “jump start” arthritis and additional exogenous IL-1β was not needed to maintain arthritis in this mast cell-deficient strain (Nigrovic et al., 2007). This suggests that IL-1β was being supplied by another cell type in the model, which we suspect was the neutrophil. This is in contrast to what we found for Ltb4r1+/− mice, where repeated doses of IL-1β were needed to maintain arthritis, suggesting an ongoing requirement for neutrophil-derived IL-1.

In addition to expressing IL-1β, we found that neutrophils also directly deliver chemokines active on themselves into the joint, adding another layer of complexity in the way their recruitment is regulated. Neutrophils directly participated in their own recruitment by expressing the CXCR2 ligand CXCL2 and to a lesser extent the CCR1 ligand CCL3, and indirectly by delivering IL-1β to the joint, thereby inducing the production of neutrophil-active chemokines from structural cells of the joint. In response to IL-1β, FLS produced CXCL1 and CXCL5, EC produced CXCL1, and MØ produced CCL9. A further complexity in the regulation of neutrophil recruitment was the observation that the expression of different neutrophil-active chemokines was regulated differentially over the course of disease. Of the CCR1 ligands, CCL4 was expressed at very early time points and from a leukocyte in the joint other than the neutrophil, whereas CCL3 and CCL9 were expressed slightly later. CXCR2 ligand expression peaked at later time points, comprised of CXCL2 from neutrophils and CXCL5 and CXCL1, both IL-1β-inducible chemokines, from synovial tissue cells. These findings suggest that different temporal and spatial patterns of CCR1 and CXCR2 ligand expression contribute to the functional usage of these two receptors and imply that despite similar cellular distribution, CCR1 and CXCR2 have nonredundant roles in vivo.

By using mice deficient in CCR1, CXCR2, and both CCR1 and CXCR2, we found that CCR1 plays an important role in the initiation of arthritis, whereas CXCR2 was critical for the maintenance phase of arthritis. In contrast to the many descriptions of partial inhibition of neutrophil recruitment with inhibition or deletion of CCR1 or CXCR2 individually, we demonstrated that deletion of both CCR1 and CXCR2 completely inhibited neutrophil recruitment and thus the development of arthritis. These data suggest that effective inhibition of neutrophil recruitment to sites of inflammation and infection in humans may require the inhibition of more than one neutrophil chemokine receptor. However, the analogous receptors that mediate this process in humans remain to be determined because the neutrophil chemokine system is not completely orthologous between mice and humans. Of note, in humans, CXCR1 and CXCR2 are the two main chemokine receptors constitutively expressed on neutrophils whereas CCR1 is expressed on human neutrophils only after stimulation with inflammatory mediators (Hartl et al., 2008). In contrast, CCR1 and CXCR2 are the two main inflammatory chemokine receptors constitutively expressed on murine neutrophils.

The differential requirement for BLT1 and CXCR2 is also reflected in their differential expression on the surface of PMNs as they travel from the bone marrow into the inflamed joint. During the course of inflammatory arthritis, BLT1 is downregulated on PMNs that have entered the joint by high concentrations of LTB₄ that are present in the arthritic joint in the model (Chen et al., 2006). Ligand-induced receptor downregulation and internalization has been well described for BLT1 (Goldman and Gotz, 1984). In contrast, the increase in CXCR2 surface expression on joint PMNs compared to BM PMNs may reflect the preferential recruitment of CXCR2 expressing PMNs once inflammation in the joint is initiated, which would be consistent with CXCR2 playing an important role in PMN recruitment at later time points. The apparent absence of CXCR2 downregulation on PMNs in the joint may be a reflection of insufficient local joint concentrations of CXCR2 ligands to induce receptor internalization, which have been reported to be 10-fold higher than concentrations needed for CXCR2-mediated chemotaxis (Rose et al., 2004).

Thus, we have found that a cascade of diverse mediators that include a lipid (LTB₄) and its chemoattractant receptor (BLT1), a cytokine (IL-1β), and chemokines active on CCR1 and CXCR2 collaborate to control neutrophil trafficking into the joint and the development of arthritis in this model. This model is likely to be incomplete, because we still have not accounted for how the very first neutrophils enter the joint to become activated by immune complexes to release LTB₄ and IL-1. We know that immune complex deposition occurs in Ltb4r1−/− mice after serum transfer (Kim et al., 2006), so it is possible that local complement activation could play such an early role. The complement receptor C5aR is required for arthritis development in this model and is a functional chemoattractant receptor expressed on murine neutrophils, and thus may play a role upstream of BLT1 and the chemokine receptors by attracting the first neutrophils into the joint after serum transfer. In preliminary experiments, however, we have found that the transfer of WT neutrophils into C5ar1−/− mice did not restore arthritis development, suggesting that C5aR may be required on cell types.
other than or in addition to the neutrophil in this model. Thus, at present we cannot determine precise role of C5aR for neutrophil trafficking in the model.

Although the K/BxN serum transfer model shares many important features with human rheumatoid arthritis (RA), there exist some important differences in autoantibody specificities and cytokine dependence. However, significant parallels between the two diseases speak to certain underlying pathologic mechanisms of inflammatory arthritis. Deposition of anti-GPI as immune complexes in the joint is critical for inflammation in the K/BxN model, and although anti-GPI is not a significant autoantibody in human RA, synovial fluid is enriched in immune complexes, including antibodies against citrullinated antigens, which probably contribute to joint inflammation (Snir et al., 2010). The success of therapy directed against TNF-α and the relative failure of anti-IL-1β therapies in treating human RA has been pointed out as another important discrepancy between the model and the human disease. However, the K/BxN serum transfer model has some partial dependence upon TNF-α (Ji et al., 2002b), and the lack of success of IL-1β therapy for rheumatoid arthritis may instead reflect suboptimal pharmacokinetics of currently available anti-IL-1β therapies in the treatment of arthritis, as shown by the fact that IL-1β is highly expressed in human rheumatoid synovial samples (Firestein et al., 1990). Furthermore, systemic anti-IL-1 therapies have been therapeutically beneficial in certain forms of arthritis, such as juvenile idiopathic arthritis and Still’s disease (Fitzgerald et al., 2005; Pascual et al., 2005), suggesting that some forms of human arthritis may be more dependent on IL-1 than others.

We suspect that “cascades of chemoattractants” may be a general principle for how these large families of diverse molecules with overlapping functions in vitro collaborate to control leukocyte trafficking in vivo. Our data described here in immune complex-induced arthritis and our prior studies in antigen-induced asthma support a model where lipid mediators function early to initiate inflammatory cell recruitment. This first wave of cells then amplifies recruitment by directly releasing chemokines in situ and by inducing the production of chemokines from tissue-resident cells through the release of cytokines. The nonredundant collaborative roles of lipid mediators and chemokines in orchestrating leukocyte trafficking in vivo may relate to differences in their production and metabolism. Lipid mediators are produced rapidly and because of their short half life are likely to act locally, whereas chemokines are produced more slowly and are capable of acting at farther distances because of their longer half-life and have even been shown to communicate with the bone marrow to mobilize responding cells into the circulation (Serbina and Pamer, 2006). It will be of interest to explore the relationship between lipid mediators, chemokines, and complement further in the control of neutrophil trafficking in this immune complex-mediated model.

**EXPERIMENTAL PROCEDURES**

**Mice**

K/BxN and Ltb4r1−/− mice were generated and maintained in our laboratory as described (Kim et al., 2006). Il1α−/− Il1b−/− mice were obtained from Y. Iwakura (Horai et al., 1998) (University of Tokyo), Cxcr2−/− mice (Cacalano et al., 1994) in the C57BL/6 background were obtained from A. Richmond (Vanderbilt University). Cxcr2−/− mice were generated by crossing Cxcr2+/− mating pairs in our laboratory. Ccr1−/− (Gao et al., 1997) and Fcer1g−/− (Takai et al., 1994) mice in the C57BL/6 background were purchased from Taconic Farms, Inc., and wild-type C57BL/6 mice were purchased from the National Cancer Institute. Ccr1−/−Cxcr2−/− mice were generated by crossing Ccr1−/− and Cxcr2−/− mice, and genotypes were confirmed by PCR. All experiments were performed according to protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

**Serum Transfer and Arthritis Scoring**

Pooled serum from 8-week-old arthritic K/BxN mice was transferred into recipient mice (150 μl) intraperitoneally (i.p.) on days 0 and 2. Ankle swelling was measured with a pocket thickness gage (Mitutoyo). Clinical scoring for each paw was measured 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.

**IL-1β Administration**

Murine recombinant IL-1β (mIL-1) (R&D) contained bovine serum albumin (BSA) from Sigma-Aldrich as a carrier protein (CP) (50 μg of CP per 1 μg of mIL-1). mIL-1 was reconstituted at 10 μg/ml in sterile PBS containing 0.1% of the same BSA that was used as its CP. mIL-1→ Ltb4r1−/− mice received mIL-1 (2.5 μg) i.p. on days 0, 1, and 2, whereas CP → Ltb4r1−/− mice received an equivalent amount of CP (125 μg) in sterile PBS + 0.1% BSA i.p. on these 3 days. On days 0 and 2, mIL-1 or CP was given 4 hr after K/BxN serum transfer.

**Histological Scoring and Immunohistochemical Staining**

Dissected ankles were fixed in 4% neutral buffered paraformaldehyde, demineralized in modified Kristen’s solution, and stained with hematoxylin and eosin (H&E). Histological scoring of inflammation, cartilage erosion, and bone erosion were scored as follows: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe according to the criteria as described (Petit et al., 2001). For immunohistochemical staining, decalcified paraffin ankle sections were stained with Vectastain ABC peroxidase kits and NovaRED substrate (Vector Labs) with CXCL1 and CXCL2 antibodies (R&D Systems) as primary antibodies. Negative controls had no primary antibody applied, and immunostained sections were counterstained with Mayer’s hematoxylin.

**RNA Isolation and Quantitative PCR**

RNA of the synovial tissues and synovial fluid and their subsequent qPCR analysis was performed as described (Kim et al., 2006).

**BM PMN Adaptive Transfer**

BM PMNs were isolated according to established protocols as described (Kim et al., 2006). A total of 5 × 10^6 PMNs in HBSS were injected into 8-week-old recipient mice via tail vein on day 0 and 150 μl K/BxN serum was injected via tail vein on days 0 and 2.

**Immumagnetic Isolation of PMNs**

BM and SF PMNs were used for RNA and protein isolation and all in vitro neutrophil experiments were isolated with an immunomagnetic separation strategy. Freshly harvested BM or SF leukocytes were first stained with phycoerythrin-conjugated Ly6G antibody (BD Biosciences) and PMNs were then isolated with EasySep PE selection kits (Stem Cell Technologies) as primary antibodies. Negative controls had no primary antibody applied, and immunostained sections were counterstained with Mayer’s hematoxylin.

**Flow Cytometry**

Cells were blocked with 2.4G2 anti-FcγRIIIa/IId receptor (BD Biosciences) and stained with the following antibodies: FITC-conjugated anti-murine Ly6G (Becton Dickinson), APC-conjugated anti-murine CD11b (eBiosciences), biotinylated anti-murine BLT1 (3D7, mouse IgG1 generated in BLT1 KO mice; B.H., unpublished data), or PE-conjugated anti-murine CCR2 (R&D) or control biotinylated mouse IgG1 (R&D) for BLT1 mAb or control PE-conjugated rat-IgG2a (R&D) for CCR2. After washing, BLT1 and its isotype control mAb were incubated with PE-coupled anti-murine BLT1 (3D7, mouse IgG1 generated in BLT1 KO mice; B.H., unpublished data), or PE-conjugated anti-murine CXCR2 (R&D) or control biotinylated mouse IgG1 (R&D) for CXCR2. After washing, BLT1 and its isotype control mAb were incubated with PE-streptavidin. Cytfluorometry was performed with a FACS Calibur Cytometer (Becton Dickinson) and analyzed with CellQuest or FlowJo software.
Flowjo software. PMNs were identified as Ly6G and CD11b double-positive cells in the granulocyte gate of forward and side scatter plots.

Detection of Pro-IL-1β by Immunoblot
SF leukocytes were isolated from the livers of K/BxN transgenic mice with 50 μl PBS, and BM was isolated from the femoral and tibial bone marrow. BM and SF PMNs were purified with EasySep kits and lysed immediately with lysis buffer (150 mM NaCl, 1 mM CaCl₂, 25 mM Tris-Cl [pH 7.4], and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Nutley, NJ) and EDTA (1 mM), NaF (20 mM). Thirty microgram total protein/lane were used for immunoblot analysis. Protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad). Pro-IL-1β was detected with a primary rabbit polyclonal antibody (Abcam) and detected with a horseradish peroxidase-labeled secondary antibody (Bio-Rad) and a chemoluminescence detection kit (Amer sham). Blots were stripped and reprobed for β-actin with a monoclonal antibody (Abcam) and a horseradish peroxidase-labeled secondary antibody (Bio-Rad).

Detection of IL-1β Release by ELISA
Immunomagnetically purified PMNs from BM and joints of K/BxN transgenic mice were incubated at a concentration of 2 × 10⁶/ml in RPMI, 10% FCS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomyocin at 37°C, 5% CO₂ for 21 hr. Cell-free culture supernatants were harvested and assayed for IL-1β (eBioscience) and LTβ (R&D) content by ELISA. The BLT1 inhibitor CP-105,696 was a generous gift from Pfizer. CP-105,696 (10 mM in DMSO) was added to cell cultures at a final concentration of 10 μM. All samples were adjusted to a final DMSO concentration of 0.1%.

Detection of Cytokines and Chemokines by Multiplex Multi-Analyte Profiling
BM PMNs were immunomagnetically purified and lysed as described above. Ankle joints were repeatedly flushed with PBS containing 2% FCS and 1 mM EDTA until the recovered joint lavage fluid was relatively acellular. The joint lavage fluid was centrifuged at 800 × g for 5 min, the supernatant was reserved, and the recovered volume was recorded. The remaining leukocyte cell pellet was then used for immunomagnetic PMN purification. The number of purified PMNs recovered in each individual BM and SF sample was calculated by flow cytometry with Polysan Polystyrene 15 μm Microspheres (Polysciences) as a reference standard. The resulting purified BM or SF PMN sample was lysed with protein lysis buffer as described above. 25 μl of BM and SF PMN lysates and supernatant from joint fluid lavage were assayed for cytokines and chemokines with a Multiplex Multi-Analyte Profiling mouse chemokine/cytokine panel (Millipore). Samples were run on a Luminex 200 machine (Luminex Corporation) and results were analyzed with Upstate BeadView software (Upstate Cell Signaling).

Immune Complex Stimulant of PMNs
Immmobilized immune complex (IC) were formed with human serum albumin (HSA) and rabbit polyclonal anti-HSA IgG Abs (Sigma-Aldrich) on high-binding 96-well ELISA plates (Corning) with 20 μg/ml HSA in 50 mM carbonate/bicarbonate buffer (pH 9.6) overnight. Afterwards, wells were washed with PBS and blocked for 1 hr with 10% FCS in PBS. Wells were washed again and incubated with HSA Abs at a 1/400 dilution in PBS and PBS alone as control for 6 hr. After another wash, 100 μl of 10⁶ BM PMNs/ml for RNA harvest and 100 μl of 5 × 10⁶ BM PMNs/ml supernatant harvest were incubated for 3 hr and 20 hr, respectively.

Primary Murine Cell Isolation and Cytokine Stimulation
All cells were derived from C57Bl/6 mice. For FLS, dissected ankle tissues were digested in Type IV collagenase (Worthington Corporation) and adherent cells maintained in DMEM with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. FLS monolayers were cultured until confluent and used between the fourth and sixth passages. Murine cardiac ECs were isolated from cardiac tissue digested in 0.2% (w/v) collagenase (Roche) and immunomagnetically double-selected via PECA M-1-coated- and ICAM-2-coated Dynal beads (Dynal Corporation). ECs were grown in DMEM containing 20% FCS, 100 μg/ml porcine heparin, and 100 μg/ml EC growth supplement (Biomedical Technologies) in gelatin-coated tissue-culture flasks. Bone marrow-derived MØ were grown on non-tissue culture-treated Petri dishes in RPMI with 10% FCS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomyocin in the presence of 10 ng/ml M-CSF (Peprotech). After 7 days, MØ were replated at a concentration of 150,000 cells/ml and stimulated with 10 ng/ml of murine IL-1β or TNF-α (R&D) for 4 hr and 16 hr, and cells were harvested for RNA with RNasea kits (QIAAGEN). Confluent layers of FLS and EC were each incubated with 10 ng/ml IL-1β or TNF-α in their growth media and similarly harvested.

Statistical Analysis
All data are presented as mean ± SEM. Comparisons were analyzed for statistical significance by two-tailed Student’s t test and one-way ANOVA with GraphPad Prism software, with p < 0.05 considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and one table and can be found with this article online at doi:10.1016/j.immuni.2010.07.018.

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