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Deficiency of CXCR2, but Not Other Chemokine Receptors, Attenuates Autoantibody-Mediated Arthritis in a Murine Model

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Objective. **Chemokines coordinate leukocyte trafficking in homeostasis and during immune responses. Prior studies of their role in arthritis have used animal models with both an initial adaptive immune response and an inflammatory effector phase. We undertook analysis of chemokines and their receptors in the effector phase of arthritis using the K/BxN mouse serum–transfer model.**

Methods. **A time-course microarray analysis of serum-transferred arthritis was performed, examining ankle tissue, synovial fluid, and peripheral blood leukocytes. Up-regulation of chemokines was confirmed by quantitative reverse transcriptase–polymerase chain reaction. The functional relevance of chemokine induction was assessed by transferring serum into mice deficient in CCR1–7, CCR9, CXCR2, CXCR3, CXCR5, CX_3CR1, CCL2, or CCL3. Further mechanistic analysis of CXCR2 involved treatment of arthritic mice with a CXCR2 antagonist, bone marrow (BM) cell transfers with $CXCR2^{+/-}$ and $CXCR2^{-/-}$ donors and recipients, flow cytometry of synovial cells, and competition experiments measuring enrichment of CXCR2-expressing neutrophils in arthritic joints of mice with mixed $CXCR2^{+/+}$ and $CXCR2^{-/-}$ BM cells.**

Results. **Gene expression profiling revealed up-regulation of the CXCR2 ligands CXCL1, CXCL2, and CXCL5 in the joint in parallel with disease activity. $CXCR2^{-/-}$ mice had attenuated disease relative to $CXCR2^{+/-}$ littermates, as did mice receiving the CXCR2 inhibitor, while deficiency of other chemokine receptors did not affect arthritis severity. CXCR2 was required only on hematopoietic cells and was widely expressed on synovial neutrophils. CXCR2-expressing neutrophils were preferentially recruited to arthritic joints in the presence of CXCR2-deficient neutrophils.**

Conclusion. **CXCR2 (but not other chemokine receptors) is critical for the development of autoantibody-mediated arthritis, exhibiting a cell-autonomous role in neutrophil recruitment to inflamed joints.**

Chemokines are a family of >50 small chemotactic proteins, divided into 4 structural subfamilies (CC, CXC, C, and CX_3C). They have wide-ranging roles in immunity, including the organization of lymphoid organ architecture; homeostatic trafficking of naive lymphocytes; migration of T and B cells to functional niches within lymphoid organs (such as germinal centers) during adaptive immune responses; constitutive trafficking of mast cells, monocyte/macrophages, and memory T cells to uninflamed tissues; and leukocyte recruitment to sites of inflammation (1). Chemokines act by increasing integrin-mediated leukocyte adhesion to endothelium, thereby facilitating leukocyte extravasation, and by establishing ordered gradients that direct leukocyte movement within tissues. Certain chemokines have additional functions such as promoting angiogenesis, inducing leukocyte degranulation, and activating cells to secrete

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mediators of inflammation. Chemokines exert their biologic activity via binding to G protein–coupled receptors; >20 are known, classified according to their ligand subfamily (CCR, CXCR, XCR, and CX_3CR). Considerable redundancy exists in chemokine–chemokine receptor pairings, with most chemokine receptors binding multiple chemokines and many chemokines binding to more than one receptor.

There has been extensive interest in characterizing the function of chemokines and their receptors in rheumatoid arthritis (RA). Numerous chemokines have been identified in the synovium and synovial fluid (SF) of RA patients, including, but not limited to, CCL2–5, CCL8, CCL19–21, CXCL1, CXCL5–10, CXCL12, CXCL13, CXCL16, CX_3CL1, and XCL1 (2,3). Synovial macrophages and fibroblast-like synoviocytes are thought to be the primary sources of chemokines in RA tissue, although other cells such as neutrophils, mast cells, lymphocytes, and endothelial cells also contribute to the chemokine milieu. Most chemokine receptors are expressed in RA synovium, generally on multiple cell types; these include CCR1–7, CXCR1–6, CX_3CR1, and XCR1 (2,3). There are only limited data on chemokine and chemokine receptor inhibition in RA. These consist of results from small clinical trials of CCR1, CCR2, and CCL2 inhibitors, with only a nonsignificant trend toward improvement in the trial of the CCR1 antagonist (3–5).

Multiple chemokines and chemokine receptors have been implicated in animal models of arthritis on the basis of experiments employing inhibitory antibodies, pharmacologic antagonists, and/or gene-targeted mice, although we are unaware of any model that has been comprehensively examined. For collagen-induced arthritis (CIA), blockade of CCL2, CCL3, CXCL2, CXCL13, CXCL16, CCR1, or CXCR4 inhibited disease, whereas genetic deficiency of CCR2 exacerbated it (2,6–10). Adjuvant-induced arthritis was ameliorated by blockade of CCL2, CCL5, CXCL1, CXCL5, CXCL10, CCR2, CXCR2, or CXCR3 (2,11–14). Antigen-induced arthritis was dampened by blockade of CXCR2 or genetic deficiency of CCR7 or CXCR5 (15,16). These models are dependent on both an initial adaptive immune response and a downstream inflammatory cascade involving innate immune cells and cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (17). Hence, studies of these models leave unclear whether the primary role of chemokines is to orchestrate T and B cell responses during the initial adaptive immune response or to mediate inflammatory cell recruitment to arthritic joints during the effector phase.

In the K/BxN mouse model of arthritis, the upstream adaptive immune response required to generate arthritogenic autoantibodies can be conveniently separated from the downstream autoantibody-mediated effector phase. The initial transgenic model was discovered after NOD mice were fortuitously crossed with C57BL/6 (B6) mice carrying the KRN mouse T cell receptor transgenes (18). This receptor recognizes a peptide from a ubiquitous glycolytic enzyme, glucose-6-phosphate isomerase (GPI), presented by the NOD mouse–derived A^{g7} major histocompatibility complex molecule (17). In K/BxN mice, an autoimmune response develops, with production of pathogenic anti-GPI antibodies that induce arthritis when transferred into normal recipients. These antibodies deposit on joint surfaces and mediate arthritis by activating the alternative complement pathway and triggering Fcγ receptors (19). Neutrophils and mast cells are both required for serum-transferred arthritis, and recruitment of neutrophils is critically dependent on leukotriene B_4 (LTB_4) and its receptor, BLT1, such that disease does not develop in their absence (20–22).

Here we present a comprehensive analysis of chemokines and their receptors in K/BxN serum–transferred arthritis, coupling gene expression profiling and knockout mice. Our results highlight the contribution of chemokines that trigger CXCR2, in particular in the recruitment of neutrophils.

MATERIALS AND METHODS

Mice. B6, BALB/c, $CXCR2^{-/-}$ (N8), $CCR2^{-/-}$ (N10), $CCR5^{-/-}$ (N6), $CCL2^{-/-}$ (N10), and $CCL3^{-/-}$ (N7) mice were obtained from The Jackson Laboratory; $CXCR3^{-/-}$ (N11), $CXCR5^{-/-}$ (N2; a gift from Dr. Martin Lipp), $CCR1^{-/-}$ (N6), $CCR3^{-/-}$ (N9), $CCR4^{-/-}$ (N10), $CCR6^{-/-}$ (N20; a gift from Dr. Sergio Lira), $CCR7^{-/-}$ (N8; a gift from Dr. Martin Lipp), $CCR9^{-/-}$ (N10), $CX_3CR1^{-/-}$ (N12; a gift from Dr. Philip Murphy), and KRN-transgenic mice have been described previously (16,18,23–29). N indicates the backcross generation, including additional crosses in our facility using mice from The Jackson Laboratory. The lines used were on the B6 genetic background, except for the $CXCR2^{-/-}$ and $CCR3^{-/-}$ lines, which were on the BALB/c background. K/BxN mice were generated by crossing KRN-transgenic B6 mice with NOD mice.

Serum-transfer experiments used matched littermate controls generated from heterozygote or heterozygote/homozygote crosses, with the exception of the $CCR4^{-/-}$ and $CCR9^{-/-}$ lines, in which B6 mice from Charles River (the source of mice used to backcross these lines) were used as controls. Genotypes were assessed by genomic polymerase chain reaction (PCR). These experiments were reviewed by the Harvard Medical School Institutional Animal Care and Use Committee, protocol numbers 02956 and 03024.

Serum-transferred arthritis. K/BxN mouse serum was collected from 8-week-old K/BxN mice and pooled for each experiment. Arthritis was induced by intraperitoneal injection

of 200 μl (for microarray experiments), 150 μl (for B6 strains), 75 μl (for BALB/c strains), or 2.5 μl/gm body weight (for the CXCR2 line, due to its variable size) of K/BxN mouse serum on days 0 and 2 (further information is available at http://cbdm.hms.harvard.edu/JJacobsAA/SupplData.html). Ankle thickness was measured with a J15 micrometer (Blet). Inhibition experiments used the CXCR2 antagonist, SB-332235 (a gift from GlaxoSmithKline), administered at 50 mg/kg body weight by oral gavage daily; controls received water by oral gavage daily (15).

RNA isolation. Ankles were dissected to expose the articular cavity and repeatedly flushed with 1 m*M* EDTA in phosphate buffered saline (PBS) to collect SF. RNA was extracted from SF cells and peripheral blood leukocytes using TRIzol (Ambion) in accordance with the manufacturer's instructions. RNA was isolated from dissected ankles using a modified LiCl/urea protocol. Briefly, dissected ankle joints were digested in 6*M* urea/2% sodium dodecyl sulfate to liberate RNA into solution. Genomic DNA was sheared by homogenization, and RNA was selectively precipitated with LiCl. The resulting RNA-containing sample was treated with proteinase K, extracted with chloroform:phenol, mixed with GlycoBlue (Ambion), and precipitated with ethanol. All RNA samples were reprecipitated with NaOAc/ethanol and treated with DNase (Invitrogen).

Microarray analysis. RNA samples were amplified and biotinylated using the MessageAmp Kit (Ambion), fragmented, hybridized to U74Av2 gene chips (Affymetrix), conjugated with streptavidin–phycoerythrin (PE), and read using the Affymetrix GeneArray Scanner. Probe-level hybridization intensities were processed into expression values using the robust multichip analysis (RMA) method (30). RMA gene expression values were $\log_2$-transformed and averaged for each time point.

The magnitude of gene up-regulation or down-regulation was measured by averaging the fold change for consecutive time points and using the greatest value as a metric (2–time point fold change) (further information is available at http://cbdm.hms.harvard.edu/JJacobsAA/SupplData.html). The false discovery rate (FDR) of 2–time point fold change cutoffs was estimated using random data sets generated from Gaussian distributions. Hierarchical clustering was performed using Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/).

Quantitative reverse transcriptase (RT)–PCR. Single-stranded complementary DNA (cDNA) was generated from RNA samples using Superscript II RT (Invitrogen) in accordance with the manufacturer's instructions. The cDNA was amplified by PCR using SYBR Green Master Mix (Applied Biosystems) and the Mx3000p thermal cycler (Stratagene). Primers were designed using PerlPrimer (http://perlprimer.sourceforge.net) to meet the following criteria: spanned introns, aligned only with the intended messenger RNA (mRNA) target on BLAST search, and produced amplification products of <300 bp. Reaction curves were scored by the cycle number at which they met a preset fluorescence threshold. These values were converted into quantitative expression values using linear regression of a 1:4 dilution series of reference cDNA. Gene expression values were normalized to that of the *hprt* gene.

Bone marrow (BM) cell transfers. BM was collected from donor mice by flushing dissected tibiae and femurs with PBS. Red blood cells were lysed with ACK buffer, and a single-cell suspension was prepared by passing the BM flush over a nylon mesh filter. The BM single-cell suspension was stained with biotin-conjugated antibodies against CD3, CD4, and CD8α; treated with magnetic streptavidin-linked beads (Miltenyi Biotech); and passed through magnetic separation columns. Recipient BALB/c mice were lethally irradiated with 2 doses of 600 rads, 4 hours apart, then received 3×10^6 BM cells in Dulbecco's modified Eagle's medium transferred by tail-vein injection.

Flow cytometry. SF cells and peripheral blood leukocytes were stained with fluorophore-conjugated antibodies, then washed and analyzed on an Epics XL-MCL flow cytometer (Beckman Coulter). The following antibodies were used: fluorescein isothiocyanate (FITC)–conjugated anti-GR1 (eBioscience), PE-conjugated anti-CXCR2 (R&D Systems), and PE-Cy5–conjugated anti-CD11b (eBioscience). Analysis was performed using Expo32 (Beckman Coulter).

Statistical analysis. Disease severity and quantitative RT-PCR data are represented as the mean ± SEM. *P* values were calculated from microarray gene expression data using one-way analysis of variance. *P* values were calculated from ankle-thickening data by taking the area under the curve over 20 days for each mouse and performing Student's 2-tailed *t*-tests.

RESULTS

Gene expression profiling of K/BxN serum–transferred arthritis. A microarray analysis was performed to characterize the molecular events underlying pathology in K/BxN serum–transferred arthritis. Ankle tissue and peripheral blood leukocytes were collected at 6 time points: day 0 (baseline), day 1 (preclinical), day 3 (disease onset), day 7 (early disease), day 12 (disease plateau), and day 18 (late disease) (Figure 1A). SF cells were collected on days 3–18. Affymetrix oligonucleotide microarrays were used to generate 3 data sets, one for each sample type, that were analyzed independently. The significance of RNA expression changes was assessed using "2–time point fold change," an approach that uses temporal relationships in the time series data to discriminate true gene expression changes from random fluctuations. The ratio of the number of genes meeting a given 2–time point fold change cutoff in each of the data sets to the number in randomly generated data sets was used to estimate FDRs (Figure 1B). Differentially expressed genes were defined as those with an FDR <0.1, yielding 486 genes in ankle tissue and 492 in SF of the 12,488 probe sets on the Affymetrix chip. In peripheral blood leukocytes, gene expression changes were indistinguishable from those seen with randomly generated data (Figure 1B). Thus, the peripheral blood leukocyte data set effectively served as a negative control comparison for the gene expression changes seen in ankle tissue and SF.

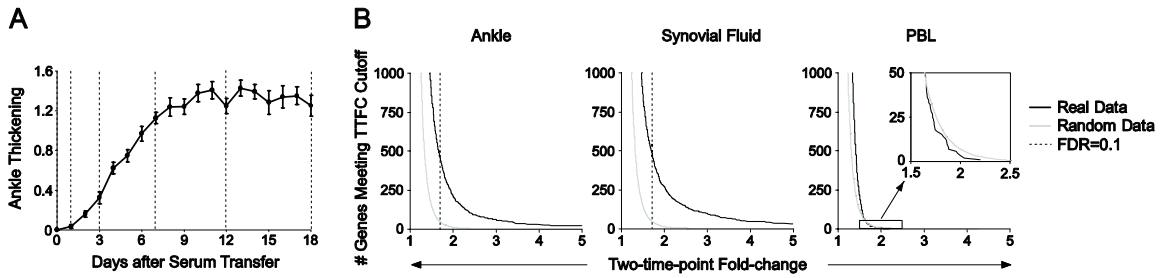

Figure 1. Microarray analysis of ankle tissue, synovial fluid (SF), and peripheral blood leukocytes (PBLs) during serum-transferred arthritis. **A,** Shown are kinetics of arthritis in C57BL/6 mice used for the microarray analysis, represented as the mean ± SEM mm ankle thickening over time. Samples were collected at the indicated time points after serum transfer (n = 3–4 samples per time point per type of sample, except n = 2 for ankle tissue on day 1 and for peripheral blood leukocytes on day 18); each sample represents pooled RNA from 2–3 mice. **B,** Two-time-point fold change (TTFC), in which fold changes from day 0 for consecutive time points (e.g., days 1 and 3, days 3 and 7) were averaged and the greatest such value used as a metric, was calculated for each gene on the Affymetrix microarray. The distribution of 2–time point fold changes for each of the 3 types of sample is shown, with the vertical axis showing the number of genes with a 2–time point fold change equal to or greater than a given 2–time point fold change plotted on the horizontal axis. The collected data ("real data") were compared with an aggregate of 100 randomly generated data sets ("random data"). Dotted lines indicate 2–time point fold change cutoff values for an estimated false discovery rate (FDR) of 0.1 in the ankle tissue and SF data sets.

The differentially expressed genes in ankle tissue and SF were further analyzed by hierarchical clustering (Figures 2A and B). Four kinetic patterns were identified in the ankle tissue data set: early-response genes (peaking on day 3, n = 25 genes), genes up-regulated in parallel with disease activity (peaking on day 7, n = 201 genes), late–up-regulated genes (peaking on day 12, n = 23 genes), and down-regulated genes (bottoming on day 7, n = 208 genes). Genes differentially expressed in the SF data set were predominantly in 2 clusters, with up-regulation (291 genes) or down-regulation (138 genes) occurring between days 3 and 7; a third, smaller cluster contained genes up-regulated between days 7 and 12 (37 genes). The ankle tissue and SF data sets overlapped significantly: 96 of the up-regulated genes in the ankle tissue data set were also up-regulated in SF

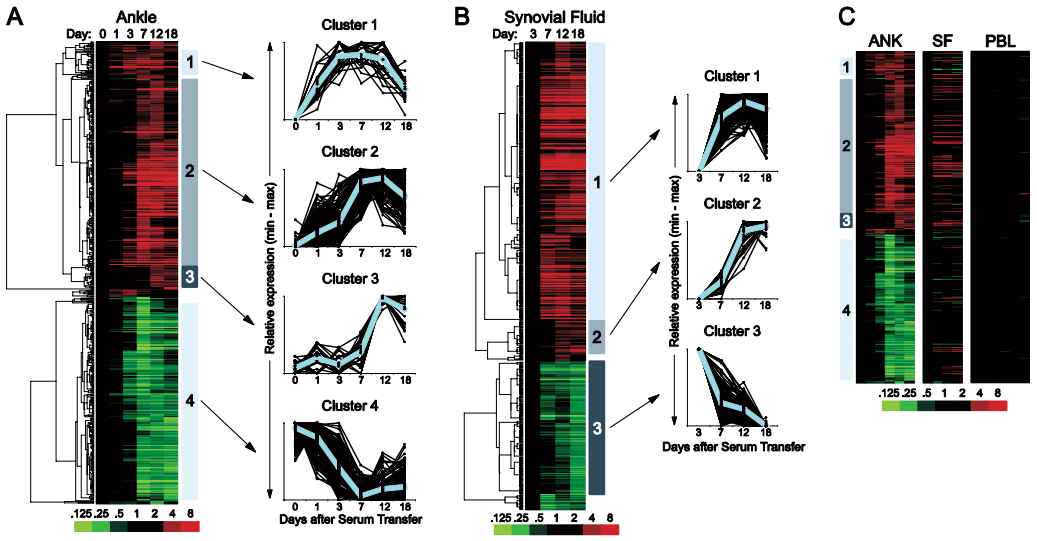

Figure 2. Hierarchical clustering of differentially expressed genes in ankle tissue and SF during serum-transferred arthritis. **A** and **B,** Genes with 2–time point fold change greater than the FDR = 0.1 cutoffs in ankle tissue **(A)** and in SF **(B)** data sets were grouped by hierarchical clustering. Fold change from day 0 is represented for each gene by a color scale from red (down-regulated) to green (up-regulated). Clusters are identified by numbered shaded bars adjacent to the hierarchically clustered data. Kinetic patterns in each cluster are represented by plotting relative gene expression values on a minimum to maximum (min–max) scale, with a blue trendline showing average minimum to maximum values for the cluster. **C,** Gene expression patterns of the 486 differentially expressed ankle tissue genes were compared across the ankle tissue (ANK), SF, and peripheral blood leukocyte data sets. Note the lack of up-regulation or down-regulation of these genes in peripheral blood leukocytes as compared with the visibly increased expression in SF of many up-regulated ankle tissue genes. See Figure 1 for other definitions.

Table 1. Selected differentially expressed genes in ankle tissue during serum-transferred arthritis*

Cluster, category, gene	TTFC	*P*
1		
C-type lectin		
DCIR	2.0	6×10^{-5}
Chemokine		
CCL2	8.6	8×10^{-6}
CCL6	1.8	0.002
CCL7	4.5	2×10^{-6}
CCL8	2.6	0.022
CCL9	2.0	0.015
CXCL12†	1.9	0.008
Chemokine receptor		
CCR2	2.1	0.008
Cytokine receptor		
IL-4Rα	1.9	0.030
Eicosanoid synthesis		
PGE_2 synthase 3	1.7	0.026
Signal transduction		
SOCS3	3.4	0.006
2		
Acute-phase reactant		
SAA2	1.9	0.002
SAA3†	49.8	2×10^{-9}
Angiogenesis		
Angiopoietin-like 4†	2.4	0.001
HIF-1α	2.8	6×10^{-4}
Cell adhesion		
SELPLG	1.7	0.005
VCAM-1†	3.5	0.001
VE-cadherin	2.0	0.035
Cell cycle		
Cdc2a†	2.4	5×10^{-5}
Cdc3a	1.7	2×10^{-4}
Cdc20	1.8	0.001
Cyclin B1†	1.9	0.060
Topoisomerase IIα	3.2	8×10^{-6}
Chemokine		
CXCL1†	6.4	4×10^{-4}
CXCL2	4.3	4×10^{-6}
CXCL5†	7.4	2×10^{-5}
Chemokine receptor		
CCR5	3.4	5×10^{-4}
Complement		
Clr†	1.7	0.017
Complement receptor		
ClqR1	2.0	6×10^{-6}
Cytokine		
IL-1β	3.4	6×10^{-5}
Osteopontin	5.0	4×10^{-5}
RANKL†	2.4	1×10^{-4}
Cytokine inhibitor		
IL-1ra	6.0	1×10^{-6}
Cytokine receptor		
IL-1R1†	1.9	0.016
IL-13Rα1	3.3	1×10^{-4}
ST2 (IL-1 receptor–like 1)†	4.8	2×10^{-7}
TNFR p75	1.8	0.021
Cytoskeleton		
Actinin a1†	2.3	6×10^{-4}
MARCKS	2.7	0.009
Extracellular matrix		
Aggrecan	3.3	1×10^{-5}
Collagen Vα2†	1.9	0.011
Lumican†	3.4	0.061
Osteoblast-specific factor 2†	3.9	0.003
Tenascin C†	5.0	8×10^{-7}
Thrombospondin 1	2.9	0.013
Versican	2.5	0.005
Growth factor		
WISP-1†	3.7	9×10^{-10}
Histamine		
Histidine decarboxylase	2.8	4×10^{-4}
L-arginine metabolism		
Arginase 1	5.9	0.002
Macrophage receptor		
Macrophage C-type lectin	9.9	6×10^{-8}
MSR1	2.4	2×10^{-5}
Protease		
Cathepsin S	2.2	0.037
MMP3†	14.6	9×10^{-8}
MMP9†	5.9	2×10^{-4}
MMP13†	16.5	1×10^{-9}
Protease inhibitor		
SLP1	13.4	2×10^{-10}
TIMP1†	5.0	4×10^{-7}
S100 proteins		
S100A8	7.2	5×10^{-8}
S100A9	8.3	8×10^{-7}
Signal transduction		
ROR2†	1.8	3×10^{-5}
SOCS2†	1.8	0.014
3		
Cytokine inhibitor		
IL-1R2	1.7	0.009
Cytoskeleton		
Synaptopodin†	2.0	0.007
Extracellular matrix		
Collagen Vα1†	1.7	0.005
Class I MHC		
H2-D1†	1.8	0.008
Growth factor regulation		
IGFBP4†	1.8	0.017
4		
Antioxidant		
Aldehyde dehydrogenase 2	1.9	0.005
Glutathione peroxidase 3	2.7	0.001
Glutathione S-transferase μl	1.8	0.002
Paraoxonase 2	1.8	0.012
Cell adhesion		
Cadherin 13	3.1	9×10^{-5}
Integrin α6	2.0	0.028
Integrin β4	1.7	0.004
Complement factor		
Factor D	2.8	2×10^{-4}
Complement inhibitor		
C1 inhibitor	1.8	0.015
Clusterin	2.5	0.004
Decay-accelerating factor 2	2.4	0.039
Eicosanoid synthesis		
LTC_4 synthase	3.1	0.002
PGI_2 synthase	2.4	4×10^{-4}
Extracellular matrix		
Cartilage intermediate layer protein 2	3.4	4×10^{-6}
Chondroadherin	2.4	9×10^{-5}
Fibromodulin	2.4	0.002
Osteocalcin	4.2	2×10^{-6}
Tenascin XB	3.3	0.003
Growth factor		
BMP4	1.7	3×10^{-5}
BMP6	1.7	4×10^{-4}
Connective tissue growth factor	2.2	0.013
Growth factor receptor		
Epidermal growth factor receptor	1.7	0.008
Lipid metabolism		
Adiponectin	2.0	0.003
Apolipoprotein D	1.8	0.001
Hormone-sensitive lipase	1.9	0.001
Lipoprotein lipase	1.9	0.003
Monoglyceride lipase	2.0	0.001
PPARγ	1.9	0.046
Protease		
Cathepsin F	2.9	0.002
Signal transduction		
ERK6	1.7	2×10^{-5}
R-ras	1.8	0.012
RasD1	2.4	0.006

* Selected genes from each of the 4 clusters of differentially expressed ankle tissue genes are shown, classified according to functional category. Values for 2–time point fold change (TTFC) and *P* values are given for each gene. The $P = 0.05$ significance level adjusted using the Bonferroni correction is $P = 1 \times 10^{-4}$.

† Also up-regulated in synovial fluid.

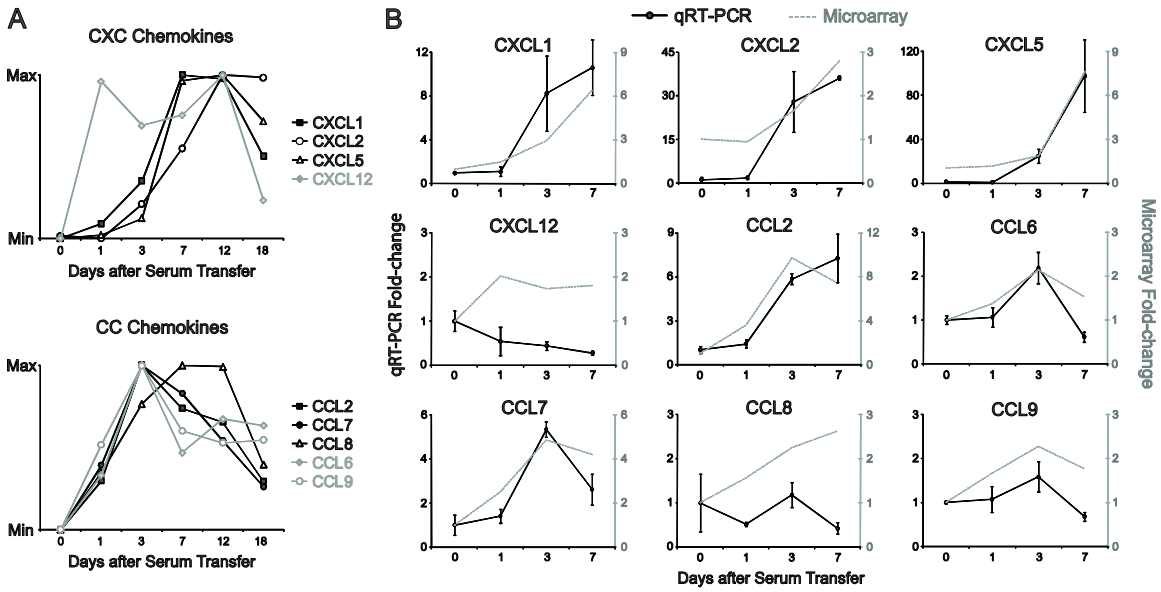

Figure 3. Chemokine gene expression in ankle tissue during serum-transferred arthritis. **A,** Microarray data for up-regulated chemokines in ankle tissue are shown in a minimum–maximum (min–max) projection, with CXC chemokines in the upper panel and CC chemokines in the lower panel. The CXC chemokines are divided into CXCR2 ligands (black) and CXCR4 ligand CXCL12 (gray). The CC chemokines are divided into CCR2 ligands (black) and CCR1 ligands (gray). **B,** Gene expression of chemokines up-regulated in the microarray analysis was assessed by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) of independent ankle tissue RNA samples collected on days 0, 1, 3, and 7 (n = 3). Quantitative RT-PCR data were compared with microarray data for each chemokine. For quantitative RT-PCR, gene expression is represented as the mean ± SEM fold change from day 0; microarray data are represented as the fold change from day 0.

(Figure 2C). The differentially expressed genes in both data sets represent diverse functional categories including cytokines, chemokines, proteases, extracellular matrix components, and adhesion molecules (Table 1) (further information is available at http://cbdm.hms.harvard.edu/JJacobsAA/SupplData.html). Similar categories of differentially expressed genes have been reported in a microarray analysis comparing synovial tissue from RA patients with synovial tissue from osteoarthritis patients (31).

CXCR2 ligands strongly up-regulated during serum-transferred arthritis. Chemokines were prominent among the genes up-regulated in ankle tissue and SF. Since both sample types shared similar differentially expressed chemokines and ankle tissue could be sampled from day 0, we focused our analysis of chemokine gene expression on the ankle tissue data set. Chemokines showed distinct expression patterns according to whether they were CC chemokines (CCL2, 6, 7, 8, and 9), which were up-regulated early, or CXC chemokines bearing the ELR motif (CXCL1, 2, and 5), which were up-regulated in parallel with disease (Figure 3A). These observed patterns of chemokine gene expression were validated by quantitative RT-PCR of independently collected ankle tissue RNA samples from days 0, 1, 3, and 7. The quantitative RT-PCR data were largely concordant with the microarray results, although the magnitude of fold changes with quantitative RT-PCR was in some cases substantially greater (Figure 3B). The most highly up-regulated chemokines by day 7 were the CXCR2 ligands: CXCL1 (11-fold), CXCL2 (36-fold), and CXCL5 (97-fold). Up-regulation of CCL8 and CCL9 could not be confirmed by quantitative RT-PCR, and quantitative RT-PCR results for CXCL12 were discordant with the microarray results.

Attenuated serum-transferred arthritis in CXCR2$^{-/-}$ mice, but not in other chemokine receptor–knockout mice. The role of chemokines in arthritis was further explored by studying serum-transferred arthritis in a large panel of knockout mice. We focused on the chemokine receptors since only a few chemokine-deficient mice exist, whereas knockout mice have been generated for most chemokine receptors. Serum-transferred arthritis was induced in mice deficient in the following receptors: CCR1–7, CCR9, CXCR2, CXCR3, CXCR5, and CX_3CR1. Only CXCR2 deficiency resulted in a statistically significant difference in arthritis compared with disease in heterozygote littermate controls when using criteria corrected for multiple hypothesis testing (Figure 4). CXCR2$^{-/-}$ mice showed a distinct pattern in which early arthritis was comparable with that of controls but then rapidly began to resolve. $CX_3CR1^{-/-}$

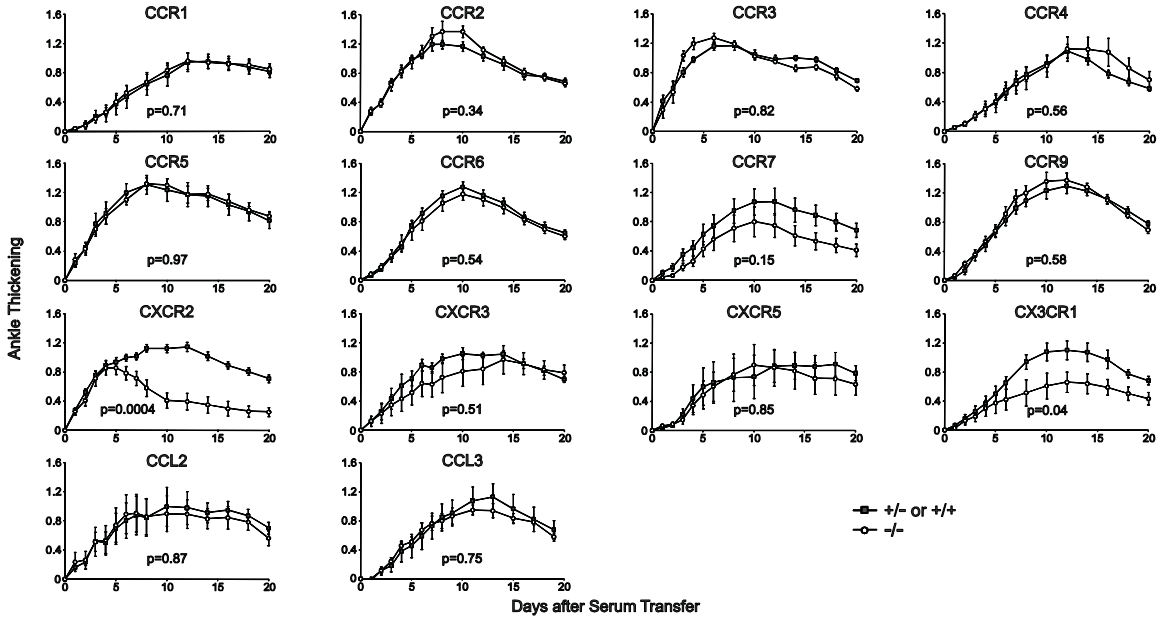

Figure 4. Attenuated course of serum-transferred arthritis in CXCR2$^{-/-}$ mice but not in other chemokine receptor–deficient animals. Serum-transferred arthritis in chemokine receptor–knockout and chemokine-knockout mice was compared with that in heterozygote littermate controls (or with that in wild-type mice for CCR4$^{-/-}$ and CCR9$^{-/-}$ mice or with that in hemizygous wild-type males for CXCR3$^{-/-}$ mice) (n = 10 mice per group for CXCR2$^{-/-}$, CCR1$^{-/-}$, CCR6$^{-/-}$, CCR7$^{-/-}$, and CX$_3$CR1$^{-/-}$ mice; n = 5 mice per group for other knockout mouse lines). *P* values were calculated for each knockout mouse line using area under the curve analysis of ankle thickening over the first 20 days. Values are the mean ± SEM mm ankle thickening. The $P = 0.05$ significance level adjusted using the Bonferroni correction was $P = 0.0036$.

mice trended toward less severe disease than that of their littermate controls, but this divergence did not reach the threshold for statistical significance when accounting for the multiple receptors tested.

Surprisingly, neither CCR1$^{-/-}$ mice nor CCR2$^{-/-}$ mice had reduced arthritis, although their ligands were up-regulated during serum-transferred arthritis. Since receptor redundancy could have accounted for the lack of effect, we investigated candidate chemokines for which knockout mice existed. CCL2, in particular, was greatly up-regulated (7–8-fold by quantitative RT-PCR) during serum-transferred arthritis and has been implicated in other arthritis models (6,11). Although CCL3 was not among the differentially expressed chemokines in the microarray analysis, it was also of interest since it is reported to be critical for anticollagen antibody–induced arthritis, an effector-phase model mechanistically similar to K/BxN serum–transferred arthritis (32). CCL2$^{-/-}$ and CCL3$^{-/-}$ mice showed disease severity equivalent to that of their heterozygote littermates (Figure 4).

We then sought to confirm the contribution of CXCR2 to serum-transferred arthritis using a pharmacologic approach. Treatment with the CXCR2 inhibitor, SB-332235, greatly reduced the severity of serum-transferred arthritis compared with that in controls (Figure 5A). The effect of CXCR2 inhibition was apparent from the onset of disease, with a reduced slope of disease progression compared with controls throughout the 10-day course of treatment. This earlier attenuation of disease compared with the CXCR2$^{-/-}$ phenotype may be due to genetic variation between mouse strains. CXCR2$^{-/-}$ mice were on the BALB/c background, whereas B6 mice were used for the inhibition experiments to facilitate comparison with the gene expression data (which derived from B6 mice) and to confirm that the CXCR2 phenotype was not specific to the BALB/c background.

CXCR2 deficiency on hematopoietic cells responsible for disease protection. CXCR2 is displayed and operates on diverse cell types, including neutrophils, monocyte/macrophages, mast cells, endothelial cells, and epithelial cells (33–35). A criss-cross BM cell transfer experiment was performed to determine whether the CXCR2$^{-/-}$ effect in this context was due to its absence on radiosensitive cells, radioresistant cells, or both. The experimental design involved BM cell transfers in all 4 combinations of CXCR2$^{+/-}$ and CXCR2$^{-/-}$ donors and recipients (Figure 5B). CXCR2 deficiency in hematopoietic cells (−/− donor → +/− recipient) reproduced the

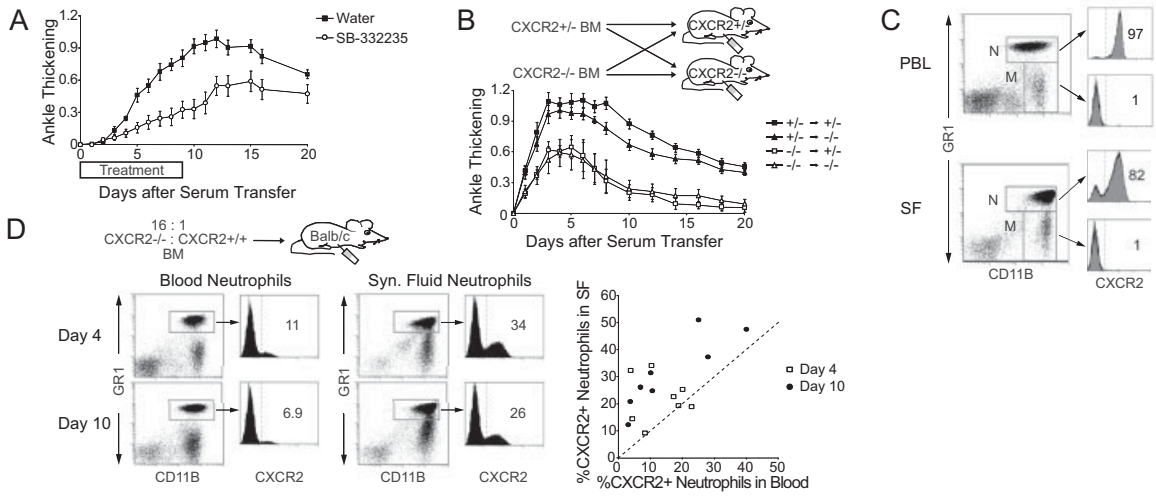

Figure 5. CXCR2 promotes neutrophil recruitment to inflamed joints in a cell-autonomous manner. **A,** C57BL/6 mice with serum-transferred arthritis received either the CXCR2 antagonist SB-332235 or water daily from day 0 to day 9 (n = 9 mice per group). Values are the mean ± SEM mm ankle thickening. $P = 0.0001$ between groups using area under the curve analysis of ankle thickening over the first 10 days. **B,** Bone marrow (BM) cell transfers were performed with all 4 combinations of $CXCR2^{+/-}$ and $CXCR2^{-/-}$ BM cell donors and recipients. Values are the mean ± SEM mm ankle thickening in each group (n = 6–9 mice per group). *P* values calculated for +/− → +/− versus −/− → +/−, +/− → −/− versus −/− → −/−, and +/− → +/− versus +/− → −/− were 0.00053, 0.0052, and 0.047, respectively. **C,** CXCR2 expression on neutrophils (N; $GR1^{high}CD11b+$) and monocyte/macrophages (M; $GR1^{low}CD11b+$) from synovial fluid (SF) and on peripheral blood leukocytes (PBLs) was assessed by flow cytometry. Equivalent results were obtained on days 4 and 10 (representative samples shown). **D,** Lethally irradiated wild-type BALB/c mice received $CXCR2^{-/-}$ and $CXCR2^{+/+}$ BM cells in a 16:1 ratio. Serum-transferred arthritis was induced after 6 weeks. SF and peripheral blood neutrophils were tested for CXCR2 expression on days 4 and 10 (representative samples are shown). The percentage of SF neutrophils expressing CXCR2 was plotted against the percentage of peripheral blood neutrophils expressing CXCR2 (n = 8 mice per group).

$CXCR2^{-/-}$ effect (−/− → −/−), demonstrating that expression of this receptor on hematopoietic cells was required. Conversely, CXCR2 display by hematopoietic cells only (+/− → −/−) was sufficient to restore normal disease kinetics, indicating that it was largely dispensable in nonhematopoietic cells. However, there was a mild decrease in arthritis severity in these mice compared with control mice (+/− → +/−), which barely achieved significance ($P = 0.047$).

CXCR2-expressing neutrophils preferentially recruited to inflamed joints in the presence of CXCR2-deficient neutrophils. CXCR2 expression was assessed on SF cells and peripheral blood leukocytes from mice with serum-transferred arthritis. Neutrophils and monocyte/macrophages are the predominant cells of the hematopoietic lineage found in SF, together comprising >90% of cells (data not shown) (18). Nearly all circulating neutrophils but few if any monocyte/macrophages expressed CXCR2 (Figure 5C), consistent with published reports (33). In SF, CXCR2 was found on 80% of neutrophils, suggesting some receptor down-regulation, and on only 1–2% of monocyte/macrophages, as in peripheral blood.

Hence, we inferred that neutrophils were a likely candidate cell type to be affected by loss of CXCR2. We performed competition experiments in which BM of lethally irradiated CXCR2-expressing hosts was reconstituted with a 16:1 mix of $CXCR2^{-/-}$ and $CXCR2^{+/+}$ BM cells, such that the resulting chimeras would have a neutrophil population consisting predominantly of knockout cells with a small number of wild-type cells (Figure 5D). Arthritis was induced, and the wild-type cells were tracked by staining for CXCR2 in peripheral blood leukocytes and SF after 4 or 10 days of arthritis. CXCR2-expressing neutrophils were enriched in SF compared with peripheral blood leukocytes at both time points, suggesting preferential recruitment. The mean odds ratio of CXCR2 expression by neutrophils in SF as compared with peripheral blood was 2.1 (95% confidence interval [95% CI] 1.1–3.9) on day 4 and 3.2 (95% CI 2.2–4.7) on day 10. This technique almost certainly underestimates the enrichment, since ~20% of wild-type neutrophils lose detectable expression of CXCR2 after migrating to the SF (Figure 5C).

DISCUSSION

Focusing on the roles of chemokines and their receptors in the effector phase of antibody-induced

arthritis, we demonstrated that CXCR2 is necessary to sustain joint inflammation. CXCR2 recognizes CXC chemokines containing the ELR motif; in mice, such chemokines include CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7 (2). Of these, CXCL1, CXCL2, and CXCL5 were up-regulated in inflamed joints during K/BxN serum–transferred arthritis. In addition, it was recently reported that CXCR2 also recognizes extracellular matrix degradation products produced by matrix metalloproteases (MMPs), and these proteases had expression kinetics similar to those of the CXC chemokines (36) (Table 1). MMPs also can enhance the potency of CXC ligands of CXCR2 through proteolytic processing, as has been shown, for instance, for MMP-9 and MMP-13 acting on CXCL2 (37). The driving force for joint expression of CXCR2 ligands may be IL-1, a cytokine expressed in parallel with disease activity that is critical for serum-transferred arthritis and that among its many activities can stimulate CXC chemokine production (38,39) (Table 1). CXCR2 ligands may mediate the neutrophil recruitment that is observed when IL-1 is introduced into tissues such as joints (40). In support of this hypothesis, neutrophil infiltration into the hippocampus triggered by transgenic IL-1 expression has been shown to be CXCR2 dependent (41).

BM cell transfer experiments revealed that CXCR2 needed to be displayed principally on hematopoietic cells. This receptor can mediate trafficking of multiple hematopoietic cell lineages involved in inflammatory arthritis, including neutrophils, monocyte/macrophages, and mast cells (33,34,36). Mast cell CXCR2 is unlikely to account for the dependence on CXCR2, since this cell type is required only prior to the onset of measurable arthritis in this system (20). It is also unlikely that defective monocyte/macrophage recruitment explains the importance of CXCR2, since only a limited number of SF macrophages expressed detectable CXCR2.

Instead, our data support a role for CXCR2 in recruitment of neutrophils to inflamed joints. This conclusion is consistent with findings in the antigen-induced arthritis model of reduced numbers of rolling, arrested, and total neutrophils in synovial tissue after CXCR2 inhibition (42,43). Those studies could not determine whether reduced neutrophil recruitment was due to a cell-autonomous requirement for CXCR2 on neutrophils themselves, or whether it was an indirect effect of decreased disease severity from CXCR2 blockade. We demonstrated that CXCR2 does indeed have intrinsic activity on neutrophils, since CXCR2-deficient neutrophils had a reduced capacity to migrate into inflamed joints when compared side-by-side with CXCR2-expressing neutrophils. A similar requirement for neutrophil CXCR2 has been demonstrated in experimental autoimmune encephalitis (44). Of note, the mild reduction in disease activity in mice lacking CXCR2 on nonhematopoietic cells may reflect a small secondary contribution of endothelial CXCR2, which can facilitate the transendothelial movement of neutrophils (35).

Although CXCR2 conferred an advantage in neutrophil migration to inflamed joints, large numbers of CXCR2-deficient neutrophils were still found in arthritic joints in the competition experiments (Figure 5D). Their presence likely reflected the activity of other neutrophil chemoattractants including LTB_4, which, along with its receptor BLT1, is required for serum-transferred arthritis and plays a sustained role after disease onset (21,22). BLT1-deficient neutrophils were abundant in the SF of BLT1-knockout mice that had received wild-type neutrophils to reconstitute arthritis susceptibility, similar to our observations on CXCR2-deficient neutrophils in the competition experiment (22). We suspect that CXCR2 and BLT1 can each mediate neutrophil recruitment in the absence of the other, but that neither mediator can fully compensate for the other. Since neutrophils are the critical source of LTB_4 in serum-transferred arthritis, CXCR2-mediated neutrophil recruitment may be amplified by LTB_4 released from the newly arrived neutrophils (21). In accord with this scenario, neutrophil migration to joints in response to intraarticular injection of CXCL1 and CXCL5 was greatly reduced by coadministration of an LTB_4 inhibitor (43). The complement receptor C5aR is also capable of mediating neutrophil chemotaxis and is required for serum-transferred arthritis (19). It may act in concert with CXCR2 and BLT1 to drive recruitment of neutrophils to inflamed joints. In human RA, CXC chemokines, LTB_4, and C5a are all present in SF, although clinical trials of BLT1 and C5aR inhibitors have yielded disappointing results (45–47). We are unaware of any reported clinical trials of a CXCR2 inhibitor in RA.

Perhaps our study's most striking finding was the surprising dearth of chemokine receptors required for the effector phase of autoantibody-induced arthritis. Like both human RA and other animal models of arthritis, the K/BxN serum–transferred disease was accompanied by up-regulation of signal chemokines, with the caveat that mRNA levels might not always correlate with protein levels. While the increased expression of ELR+ CXC chemokines in ankle tissue was reflected by reduced arthritis in the absence of their receptor, CXCR2, this correlation of induction level with disease

severity did not hold either for the receptors of other up-regulated CC chemokines or for CCL2. It is possible that receptor and/or ligand redundancy permits other receptors to take the place of any single chemokine receptor targeted. Results for certain of the receptors tested, in particular CX_3CR1, fell just below statistical significance, so we cannot exclude the possibility that they have a moderate contribution to disease that might be confirmed by further testing. Moreover, some chemokine receptors were not included in this study for technical reasons, most notably CXCR4 (the receptor for CXCL12), which could not be tested since deficiency in this molecule is lethal during embryogenesis.

However, these caveats do not explain why inhibition or deficiency of CCR1, CCR2, CCR7, CXCR3, CXCR5, CCL2, or CCL3 altered disease severity in other arthritis models but not in serum-transferred arthritis (6,9,10,14,16,32). These disparate results cannot simply be attributed to inadequate genetic targeting, since studies in other animal models used the same CCR7-, CXCR5-, and CCL3-knockout lines, and the other lines used here have previously been clearly demonstrated to lack expression of the targeted gene (16,23,24,32,48,49). The discrepancy may reflect the contributions of these chemokines and chemokine receptors to adaptive immunity, consistent with evidence of altered T or B cell activity reported in many of those studies. In CIA, increased disease severity with CCR2 deficiency was associated with enhanced titers of anticollagen antibodies, formation of rheumatoid factor (not detected in controls), and decreased activation-induced cell death (10). In a study demonstrating improvement in adjuvant-induced arthritis with anti-CXCR3 monoclonal antibody therapy, $CXCR3^{-/-}$ mouse T cells had a reduced capacity to migrate to inflamed joints in comparison with wild-type mouse T cells (14). $CCR7^{-/-}$ and $CXCR5^{-/-}$ mice with antigen-induced arthritis had reduced titers of antibodies to the inciting antigen, reduced T cell proliferation in response to antigen, and aberrant or absent lymphoid follicle formation within chronically inflamed synovial tissue (16).

CCL3 represents a special case, since it has been shown to have a role in anticollagen antibody–induced arthritis, which, like serum-transferred arthritis, is induced by passive antibody transfer (32). The primary difference between the 2 models besides their autoantibody specificity is the requirement for lipopolysaccharide (LPS) in anticollagen antibody–induced arthritis. LPS induces CCL3 and promotes neutrophil accumulation in a CCL3-dependent manner (50). It may be that, in the absence of CCL3, LPS is unable to provide enough additional proinflammatory activity to induce disease upon transfer of anticollagen antibodies, whereas antibodies alone are sufficient to induce disease in K/BxN serum–transferred arthritis.

This study demonstrates a necessary role for CXCR2 in sustaining autoantibody-induced arthritis and identifies this receptor as a second critical element by which neutrophils can be recruited to inflamed joints, alongside BLT1. Our findings highlight CXCR2 and potentially also CXCR1 (a closely related human receptor without a known murine homolog that binds CXCL6 and CXCL8; also known as IL-8) as attractive therapeutic targets for RA (2). The importance of CXCR2 in the arthritis effector phase, the stage when most patients present with disease, enhances its appeal.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Mathis and Benoist had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Jacobs, Campbell, Gerard, Mathis, Benoist.

Acquisition of data. Jacobs, Ortiz-Lopez, Gerard.

Analysis and interpretation of data. Jacobs, Gerard, Mathis, Benoist.

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