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CX3CR1 deficient mice have decreased Th17 and antigen-specific humoral responses in the collagen induced arthritis (CIA) model

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Abstract

Objective—CX3CR1 is a chemokine receptor that uniquely binds to its ligand fractalkine (FKN or CX3CL1) and has been shown to be important in inflammatory arthritis responses largely due to effects on cellular migration. In this study, we tested the hypothesis that genetic deficiency of CX3CR1 would be protective in the chronic inflammatory arthritis model, collagen induced arthritis (CIA). Because CX3CR1 is expressed on T cells and antigen-presenting cells, we additionally examined adaptive immune functions in this model.

Methods—Autoantibody formation, clinical, histologic, T cell proliferative, and cytokine responses were evaluated in DBA-1J mice deficient in (-/-) or wildtype (+/+) for CX3CR1 after immunization with heterologous type II collagen.

Results—CX3CR1-/- mice had an approximate 30% reduction in arthritis by two independent measures of paw swelling ($p<0.01$) and clinical disease score ($p<0.0001$). Additionally, CX3CR1-/- mice had an approximate 50% decrease in anti-type II collagen autoantibody formation ($p<0.05$), decreased Th17 intra-articular cytokine expression (IL-17 $p<0.01$ and IL-23 $p<0.001$), and decreased total numbers of Th17 cells in inflamed joints ($p<0.05$).

Conclusions—Deficiency of CX3CR1 is protective in inflammatory arthritis and may have effects that extend beyond migration that involve adaptive immune responses in autoimmune disease.

Introduction

Many chemokine-receptor interactions have been implicated in the inflammatory cellular trafficking of rheumatoid arthritis (RA) (reviewed in (1)). However, the promiscuity of ligand-receptor interactions within most chemokine receptor families has been difficult to overcome therapeutically in clinical trials that have targeted the blockade of an individual chemokine or its receptor in arthritis patients (2, 3). The solitary member of the CX3CR

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family, CX3CR1, is unique in that it has only one known ligand, fractalkine (FKN or CX3CL1) (4), and blockade of the CX3CL1/CX3CR1 signaling axis has been shown to be efficacious in several pre-clinical models of inflammation (reviewed in (5)). With particular relevance to RA, CX3CL1 and CX3CR1 are upregulated in inflammatory cells within the synovial tissue in rat adjuvant induced arthritis (AIA) (6), and CX3CL1 mediates T-cell dependent proliferation of synovial fibroblasts from RA patients (7). In the mouse collagen induced arthritis (CIA) chronic model, mice treated with a neutralizing antibody to CX3CL1 have lower clinical scores, improved histology, and decreased migration of adoptively transferred splenic monocytes to the joint (8). Additionally, patients with RA have increased CX3CR1+ T cells circulating in the peripheral blood (6), and increasing levels of CX3CR1+ T cells and monocytes in the synovial fluid that correlate with disease activity (6). These data suggest that CX3CL1/CX3CR1 signaling plays an important role in the trafficking and function of inflammatory cell subsets in RA.

CX3CR1 signaling is also important in the pathogenesis of inflammatory vascular disease and atherosclerosis (9-12), which is a complication from longstanding RA (13). Our group has shown that CX3CR1 deficiency is protective from intimal hyperplasia after arterial injury in mice as a result of decreased monocyte trafficking (9) and decreased dendritic cell accumulation (11) in atherosclerotic plaques. In humans, a naturally occurring gene polymorphism (CX3CR1-M280) correlates with a lower prevalence of atherosclerosis (10, 12), which could potentially be explained by reduced CX3CL1-dependent cellular adhesion in inflammatory cells expressing CX3CR1-M280 (10). These data suggest that blockade of CX3CR1 interactions may be an important therapeutic target for the treatment of RA and the inflammatory sequelae that arise from it, such as atherosclerosis.

Because CX3CR1 is predominantly expressed on T cells and antigen presenting cells (11, 14, 15), we hypothesized that adaptive immune responses may be affected beyond the migration abnormalities seen with blockade of the ligand CX3CL1 (8) in an immunization model of inflammatory arthritis (CIA). Consequently, we investigated clinical disease outcomes, autoantibody formation, T cell responses, histopathology, and cytokine responses in the CIA model comparing mice with a gene deletion of CX3CR1 (CX3CR1-/-) to that of wildtype controls (+/+). Our results suggest that inhibition of CX3CR1 may have beneficial effects in inflammatory arthritis beyond that of migration since decreased autoantibodies and pro-inflammatory Th17 responses were observed in CX3CR1-deficient animals.

Materials and Methods

Animals

All animals were bred, housed, and cared for in DLAM facilities under the approved IACUC protocol number 09-245.0 in pathogen free specific conditions.

Antibodies

Antibodies used for these experiments purchased from eBioscience (San Diego, CA) included anti-CD3 and anti-CD28 for T cell proliferation studies, and anti-CD4-eFluor 450, and anti-IFN-γ-APC for flow cytometry. Anti-CX3CR1 antibodies (R&D, Minneapolis, MN) and anti-IL-17A-PE antibodies (BD Pharmingen, San Diego, CA) were also used for flow cytometry.

Induction and evaluation of Collagen Induced Arthritis

CX3CR1-/- and wildtype controls were backcrossed 7 generations on the highly susceptible CIA background, DBA-1J (16). 8 week old male mice were immunized with Freund's adjuvant (Sigma Aldrich, St. Louis, MO), Complete on day -21 and Incomplete on

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day 0, with 100 μg per mouse of bovine type II collagen (Chondrex, Redmond, WA) in a 1:1 mixture of adjuvant and collagen injected subcutaneously into the base of the tail. Our protocol utilizes a second booster injection because 1) a single injection has more variability in disease onset, 2) using two injections, most animals achieve maximum disease by 6 weeks compared to only 40% after a single injection, and 3) a single injection results in less overall total disease severity. This protocol has been well characterized by Wooley and others (16). Mice were measured by a blinded observer for 1) a clinical disease scoring index and 2) measurement of paw swelling from baseline. Clinical disease index was performed with the following scoring system: 0=normal paw; 1=mild but definite swelling of either the ankle or digits; 2=moderate redness and swelling of an ankle ± any number of digits; 3=maximal redness and swelling of the entire paw and digits with or without ankylosis. The maximum score per paw was 3 with a total score obtainable of 12 per mouse. This scoring system has been validated by our group previously (17, 18). Paw swelling measurements were obtained by measuring the thickness of the fore- and hindlimbs at the wrist or ankle respectively. Paw swelling is presented as a change in the mean thickness of the mouse's fore- and hindlimbs (mm) from its baseline average.

Histopathology

At experiment termination, hindlimbs were fixed (4% paraformaldehyde), decalcified (formic acid), and embedded in paraffin. Serial 5 μm sections were cut and stained with hematoxylin and eosin (H&E) according to standard protocols for morphologic analysis.

Anti-collagen antibody ELISA

IgG anti-type II collagen antibodies were measured by standard sandwich ELISA (Chondrex, Redmond, WA). Autoantibodies in CIA can be assessed at many time points, but are typically analyzed between days -7 and 14, when they are most elevated (19). We chose to analyze autoantibodies before the second injection and disease onset (day -7 and day 0) to examine the rise in production of autoantibodies, which are thought to precede clinical illness and be directly pathogenic (20). Serum was collected from CIA immunized mice (days -7 and 0) by tail vein according to previously published protocols (21) and diluted 1:5,000 for autoantibody measurement by ELISA, as performed per manufacturer's instructions.

Lymph node T cell proliferation and intra-cytokine staining for IL-17 and IFN-γ

In CIA, autoantigen recognition and T cell proliferation and differentiation are initiated in the first 2 weeks of initial immunization (22). This is typically the optimal time to assess T cell proliferative responses and T cell cytokine production to *in vitro* stimulation. We isolated the draining lymph nodes (iliac and inguinal) from the site of immunization (base of the tail) where we expected to see the most potent immune response (day -7 second injection). For proliferation studies, cells were isolated in RPMI + 1% naïve syngeneic mouse sera + 10 mM HEPES + 25 units/ml heparin, and then further enriched for CD4+ T cells using the negative selection MACS mouse CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA) as per manufacturer's instructions. Enriched T cells from WT or CX3CR1-/- mice were stimulated in 96 well flat-bottomed plates using 3 conditions: 1.) with anti-CD3 (1μg/ml) + anti-CD28 (1μg/ml), 2.) with irradiated APCs from the spleen of a naïve syngeneic mouse + T cell proliferation grade bovine type II collagen (100 μg/ml) (Chondrex, Redmond, WA), or 3.) with irradiated APCs in media alone. Cultures were incubated at 37° C for 48 hrs, then 1 μCi of ^{3}H-thymidine was added to each well, and cultures were incubated an additional 15-18 hr prior to scintillation counting. For intra-cytokine staining of IL-17 or IFN-γ, lymphocytes were stimulated with anti-CD3 (5μg/ml coated plate) and anti-CD28 (1μg/ml) in 24-well cell culture for 6 hrs, and Golgi Stop (1ul/ml, BD Biosciences, San Jose, CA) was added to the culture for the last two hours. Cells

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were harvested and stained for surface CD4 and intracellular IL-17 and IFN-γ, and then analyzed by flow cytometry.

RT-PCR for intra-articular cytokine, matrix metalloproteinase, and chemokine/receptor analysis

Arthritic paws (score=2) were collected for RNA analysis on day 10, which is early onset of arthritis and were compared to naïve, unimmunized paws (score=0). All paw samples were homogenized using an OmniTH homogenizer. Total RNA was extracted with TRIZOL reagent (Invitrogen Life technologies, Carlsbad, CA) and cDNA synthesis was carried out with Superscript II reverse transcriptase (Invitrogen Life technologies) according to the manufacturer's protocol. Real time quantitative PCR (qRT-PCR) was performed using a Bio-Rad iCycler and Sensimix SYBR and Fluorscein kit (Quantace, LTD, Tauton, MA). The total volume of each reaction was 25 μl including 12.5 μl of 2X SYBR Green supermix, 0.625 μl of each primer at a concentration of 20 μM, 10.25μl of RNase-free water and 1 μl of cDNA. The PCR was carried out using the following thermocycling conditions: 95°C for 3 min, 40 cycles at 60°C for 30s, 95°C for 1 min and 60°C for 1 min. IDUA rRNA was used as a control. The following sets of primers were used: IDUA: forward-GCATCCAAGTGG GTGAAGTT, reverse-CATTGAGCAGGTCCGGATAC; FKN: forward-CGCGTTCTTCCATTTGTGTA, reverse- GTCTGTGCTGTGTCGTCTCC; MMP1; forward-AACTACATTTAGGGGAGAGGTGT, reverse-GCAGCGTCAAGTTTAACTGGAA; MMP2: forward-CGGAGA TCTGCAAACAGGACA, reverse-CGCCAAATAAACCGGTCCTT; MMP9: forward-GCGTGTCTG GAGATTCGACTT, reverse-TATCCACGCGAATGACGCT; MMP13: forward-CTTCTTCTTGTTGAGCTGGACTC, reverse-CTGTGGAGGTCACTGTAGACT; TNFα: forward-CATCTTCTCAAAATT CGAGTGACAA, reverse-TGGGAGTAGACAAGGTACAACCC; IL1-β: forward- GGT CAA AGG TTT GGA AGC AG, reverse- TGT GAA ATG CCA CCT TTT GA; IL6: forward-. CAA AGC CAG AGT CCT TCA GAG, reverse- GGA TGG TCT TGG TCC TTA GC; IL17: forward- CTC CAG AAG GCC CTC AGA CTA C, reverse- AGC TTT CCC TCC GCA TTG ACA CAG; IL23p19: forward - AGC GGG ACA TAT GAA TCT ACT AAG AGA, reverse - GTC CTA GTA GGG AGG TGT GAA GTT G -3. The RT-PCR primers that were used for IL-17 detection in the joint are specifically directed toward the family member IL-17A, which is exclusively expressed by T cells (23).

Intra-articular determination of Th17 cells

Inflammatory cells were liberated from the early inflamed joint according to our previously published protocol (18) using dissection of the joint capsule followed by Collagenase D (Sigma Aldrich, St. Louis, MO) digestion at 2 mg/ml in warmed PBS for 30 minutes. Afterward, cells were strained through a 70 micron filter to inhibit contamination with stromal cells, stained for surface CD4 expression, fixed and permeabilized with BD Cytofix/ Cytoperm (BD Bioscience, San Diego, CA), and then stained for intracellular IL-17A.

Data analysis

For clinical disease and paw swelling curves, a statistical curve-fit was used as in our previous reports (17, 18) to determine whether significant differences existed in disease over time between CX3CR1-/- mice versus wildtype controls. The advantage of this statistical test is its effectiveness for studying change. A backward selection (α=.05) procedure was used to select a linear mixed model with the best fit for the individual curves. Statistical variables included group, time, and experiment effect. The longitudinal analysis (mixed model) affords us the ability to distinguish variation that may be observed across time for a particular mouse from the variation among the group (24). The overall group effect was assessed using a likelihood ratio test (LRT_x) (α=.05). The best fit curves were plotted using

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predicted values calculated using the fixed effects from the models, averaging across experiment, which was controlled for if it was a significant (α=.05) predictor in the model using SAS, v. 9.1. For T cell proliferation, anti-collagen antibody assays, intra-articular cytokine analysis by RT-PCR, and quantification of Th17 cells in inflamed joints by flow cytometry, an unpaired two-tailed t test was used to compare the means between groups.

Results

CX3CR1-/- mice have less arthritis in CIA when compared to controls

In humans with RA, increased CX3CR1 expression on circulating T cells (6, 25) and on T cells and monocytes in the synovial fluid (6) correlates with disease activity. To investigate whether or not absence of CX3CR1 expression confers protection in inflammatory arthritis, we examined CX3CR1-/- mice and their wildtype controls in a chronic inflammatory arthritis model (CIA), which has similarities to rheumatoid arthritis in its histopathology, MHC II restriction, and waxing-waning clinical course (16). We found that animals with a targeted genetic deletion of CX3CR1 had approximately 30% less disease when compared to controls by two independent measures of paw swelling ($p<0.01$) and clinical disease severity index ($p<0.0001$) (Figure 1A, B). Although there was less inflammation in CX3CR1-/- mice (Figure 1A, B), the CX3CR1-/- animals were capable of developing erosions (Figure 1D) similar to wildtype controls (Figure 1C) by histopathology.

CX3CR1-/- mice have decreased autoantibody formation to type II collagen but similar T cell proliferative responses

CX3CR1 is expressed on monocytes (15), B cells (26), and dendritic cells (6, 27), which are antigen presenting cells important for the induction of antibody responses. Additionally, the percent of dendritic cells expressing CX3CR1 is known to increase during joint inflammation in rat AIA (6), which could affect antigen presentation and subsequent autoantibody formation. For these reasons, we hypothesized that adaptive immune responses may be affected as a result of the absence of CX3CR1 on immune cells, and effects on CIA could extend beyond the migration abnormalities previously seen with blockade of the ligand CX3CL1 (8). Consequently, we examined levels of pre-clinical anti-type II collagen autoantibodies, which are known to be pathogenic in CIA and develop prior to the onset of clinical arthritis (20). In CIA, T cell activation, proliferation, and initiation of B cell responses are initiated in the first 2 weeks of initial immunization (22); therefore, we chose to examine lymph node derived T cell proliferative responses as well as autoantibody formation at this time point. Anti-type II collagen antibody responses from CX3CR1-/- mice were approximately 50% less than that of wildtype controls on both day -7 ($p<0.05$) and day 0 ($p<0.01$) with substantially higher autoantibodies in wildtype animals as they were approaching the onset of clinical disease (day 0) (Figure 2). In contrast, no significant differences were noted in T cell activation and proliferation (Table 1).

CX3CR1-/- mice with CIA have a selective decrease in Th17 cytokines and decreased total numbers of T helper 17 (Th17) cells in arthritic paws

Pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-17, IL-23, and matrix metalloproteinases (MMPs) have been shown to play an important role in the pathophysiology of inflammatory arthritis in both humans and in animal models (28-32). Consequently, we chose to examine intra-articular cytokine production of these key pro-inflammatory mediators by RT-PCR (33) using severely inflamed joints (clinical score=2) from animals with early inflammation (day 10). MMP-1, -2, and -9 were similar between both groups (Figure 3B) in addition to CX3CL1 (Figure 3A). CX3CR1-/- mice had decreased, but not statistically significant, levels of pro-inflammatory MMP-13 (Figure 3B) and cytokines IL-6, IL-1β, and TNF-α (Figure 3A). In contrast, IL-17 levels were reduced

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>5 fold ($p<0.01$), and IL-23 was reduced >3 fold ($p<0.001$) in CX3CR1-/- mice (Figure 3A). To determine if there were fewer Th17 cells in the inflamed joints of CX3CR1-/- mice, we dissected and collagenase digested the joint capsules of severely inflamed (clinical score=2), early arthritis paws (days 14-16) from wildtype (n=10) and CX3CR1-/- (n=7) CIA mice and analyzed cellular infiltrate by flow cytometry. We chose endpoints of days 14-16 for flow cytometry as compared to day 10 for RT-PCR to ensure that protein levels of IL-17 were elevated above the limit of detection after transcriptional upregulation. CD4+ IL-17+ T lymphocytes were identified in the joints of CIA immunized mice, and CX3CR1-/- mice had a 3-fold decrease in the absolute number of articular Th17 lymphocytes as compared to wildtype mice ($p<0.05$) (Figure 4).

To further examine the relationship of CX3CR1 and IL-17 on T cells, we analyzed lymphocytes from the draining lymph nodes (iliac and inguinal) by flow cytometry and identified a small subpopulation of CX3CR1 expressing cells above background levels that also expressed CD4 and IL-17. Specifically, of the wildtype lymphocytes that stained positive for both CD4 and CX3CR1 $0.96\% \pm 0.15\%$ (n=5), we detected $5.14\% \pm 1.4\%$ of this subpopulation to also be positive for IL-17. This population was too small to reliably perform migration studies. To investigate whether CX3CR1 deficiency had a functional effect on IL-17 production during antigen presentation in the lymph nodes after immunization, we harvested draining lymph nodes (iliac and inguinal) from immunized mice 2 weeks after the initial immunization (day -7) and stimulated isolated T cells *in vitro* with anti-CD3/CD28 antibodies. CD4+ T cells were selected and analyzed for intracellular expression of IFN-γ and IL-17, and no statistical differences were seen between CX3CR1-/- mice and controls (Figure 5B). This may be due in part to the small fraction of CD4+ CX3CR1+ T cells detected over background immunostaining for CX3CR1 as compared to the total population of CD4+ T cells (Figure 5A).

Discussion

CX3CL1/CX3CR1 signaling has been established as an important pro-inflammatory chemokine receptor signaling interaction in chronic inflammatory diseases (reviewed in (5)) including RA (1, 6, 8, 34, 35) and atherosclerosis (10-12). Correlation studies have shown increased expression of CX3CL1 and/or CX3CR1 in RA (34, 35) and that they are more elevated in patients with severe disease (35). In a mouse CIA model, treatment of arthritic mice with a neutralizing antibody to the unique ligand for CX3CR1, CX3CL1, showed protection from disease that was mediated by inhibition of macrophage/monocytes trafficking to the joint (8). However, beyond impairment in macrophage/monocyte trafficking, changes in B and T cell function were not observed in these studies (8).

CX3CL1 is highly expressed on endothelial cells, intestinal epithelial cells, synoviocyte-like fibroblasts, and on some dendritic cells (6, 36-38). In contrast, its receptor CX3CR1 is expressed mostly on immune cells such as monocytes, macrophages, dendritic cells, NK cells, a subset of T cells, and recent reports that suggest a subset of B cells (11, 14, 15, 26). CX3CL1/CX3CR1 signaling activates the proinflammatory NF-kB pathway (39), and CX3CR1 deficiency is associated with decreased IL-6 and TNF-α production by macrophages and dendritic cells (40). Because of its predominant expression on immune cells and multiple implicated mechanisms of immune regulation, we examined the genetic deletion of CX3CR1 in the CIA model to determine whether or not additional mechanisms beyond that of cellular trafficking may be affected in inflammatory arthritis when the receptor, as opposed to the ligand, was targeted.

Our studies show that CX3CR1 deficiency confers protection in CIA, in part, through decreased humoral and T cell responses. Since professional antigen presenting cells (i.e.

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monocytes, macrophages, B cells, and dendritic cells) have CX3CR1 on their cell surface (9, 11, 15, 26, 27), it is conceivable that alteration of the CX3CR1+ subset of these antigen-presenting cells could lead to decreased production of autoantibodies, which are known to be pathogenic (20). Recently, Corcione et al. immunized CX3CR1-/- and wildtype mice with ovalbumin (OVA) to determine whether or not CX3CR1 deficiency had effects on antigen-specific antibody formation. Their study had findings similar to ours in that OVA-specific IgG production was decreased (26). Lymphoid follicle architecture, B cell, and T cell enumeration did not differ, thus the authors concluded that differences in autoantibodies could not be directly determined. Interestingly, a study by Bar-On et al (27) has identified a specific subpopulation of CX3CR1+ CD8α+ dendritic cells that share a gene signature overlapping with plasmacytoid dendritic cells. Since plasmacytoid dendritic cells are known for regulating B cell differentiation and antibody production (41), this CX3CR1+ dendritic cell subset may have functions that affect antibody production in inflammation.

The Th17 subset is recently recognized as an important pro-inflammatory mediator in RA (30). Neiss et al. showed that CX3CR1+ peptide-pulsed dendritic cells preferentially supported the differentiation of CD4+ Th17 cells *in vitro* in an inflammatory bowel disease model (40). Our study did not see a difference in the ability of CX3CR1-/- mice to develop Th17 skewing *in vitro*, albeit there was a trend toward decreased Th1 IFN-γ production, which would support the conclusions of Neiss et al. We also acknowledge that only a small subset of CD4+ T cells express CX3CR1 (Figure 5A) after immunization for CIA, which may have limited our ability to detect a difference in the cytokine production assays.

An additional or alternative mechanism beyond that of impaired Th17 induction in CX3CR1-/- lymph node cells could be the selective impairment of this particular T-helper subset to migrate to the joint. Although we were able to detect a subpopulation of CD4+ T cells that also expressed CX3CR1 above background levels (Figure 5A), the total numbers of IL-17+ CD4+ CX3CR1+ cells are low enough (5% IL-17+ of the 1% CD4+ CX3CR1+ lymphocytes) that this raises questions as to the accuracy and reproducibility of this measurement. The ideal experiment would be to examine CIA responses and CD4+ IL-17+ cells using the $CX3CR1^{GFP}$ reporter mice (4), which have green fluorescent protein knocked into the CX3CR1 gene locus. In this way, a more sensitive and accurate examination of the CX3CR1 expression pattern and migration of small cellular subsets that normally express this receptor (such as Th17 cells) could be more reliably ascertained and studied. Although direct migration and enumeration of CD4+ IL-17+ CX3CR1+ cells in CIA was limited, we were able to show 3-fold decreased numbers of CD4+ IL-17+ cells in the inflamed paws of CX3CR1-/- mice (Figure 4). Although altered migration is suggested in that there are fewer Th17 cells in the CX3CR1-/- inflamed paw, the evidence is indirect, and we cannot exclude the possibility of other mechanisms such as enhanced apoptosis or impaired proliferation of Th17 cells as well.

Based on our findings of decreased IL-23 in the joint, we additionally postulate that within the joint cytokine milieu, there may be environmental differences that affect local T cell cytokine secretion and function. Specifically, IL-23 is secreted by dendritic cells and induces the production of IL-17 by T cells (42). IL-23 is not needed for the *de novo* generation of Th17 cells but can augment IL-17 production from already generated Th17 memory cells (43). Consequently, decreased IL-23 within the local microenvironment of the joint may additionally affect the function of Th17 cells in CX3CR1-/- mice.

The CIA model is an approximation of human RA and does have limitations. Particularly, the inflammatory reaction is robust, and even in modest disease, extensive damage to the joint is seen at early and chronic time points. Therefore, a reduction, as opposed to elimination of disease, may not achieve statistical significance in histopathology scores in

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this model. One of our proposed mechanisms of protection is that loss of CX3CR1 impairs cellular trafficking to the joint, particularly of cells involved in the Th17 axis. However, inflammatory cells are not completely inhibited in trafficking to the joint, as seen on the histopathology sections (Figure 1), which may explain why disease is not significantly different at later time points. Additionally, matrix metalloproteinases (MMPs), which implement much of direct tissue destruction in CIA, are not different between CX3CR1-/- and wildtype mice, which could explain why bony erosions and cartilage loss are not significantly different between groups.

Our group has previously published on the importance of CX3CL1/CX3CR1 signaling in atherosclerosis, which is attributed in part to alterations in inflammatory monocyte and dendritic cell trafficking to affected lesions (9-11). Accelerated atherosclerotic disease from longstanding RA is becoming a widely recognized long-term complication (13). In a recent observational study by Pingiotti et al., peripheral blood isolated CD4+ CX3CR1+ T cells from RA patients were expanded when compared to healthy controls (34). Further, this increase in CD4+ CX3CR1+ T cells from the RA patients correlated with increased carotid intima-media thickness (IMT) and the Disease Activity Scoring system (DAS 28) in Rheumatoid Arthritis (34). These data suggest that CX3CL1/CX3CR1 blockade may have long-term benefits that extend beyond inflammatory arthritis and into prevention of early endothelial dysfunction leading to atherosclerotic disease in these patients.

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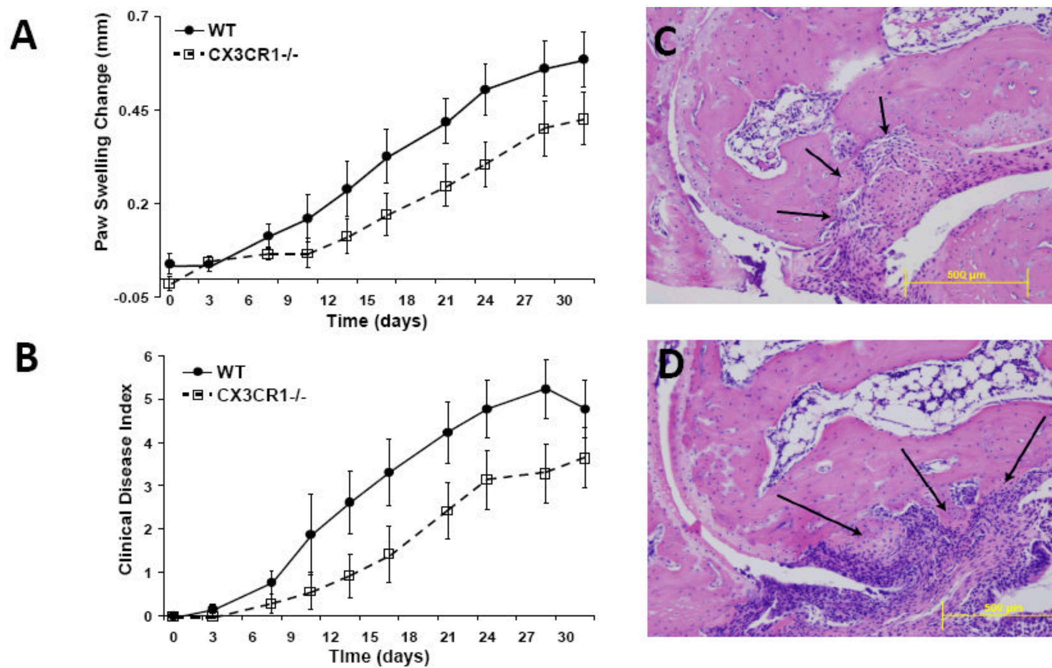

Figure 1. CX3CR1-/- mice have decreased clinical inflammation by two independent measures compared to wildtype controls in the collagen induced arthritis model (CIA)

A. CX3CR1-/- mice (n=14) have decreased paw swelling ($p<0.01$) and **B**. clinical disease assessment scores ($p<0.0001$) compared to wildtype controls (n=13) in CIA. Both wildtype **C**. and CX3CR1-/- **D**. mice have similar erosions at experiment termination on day 35 after the second booster immunization. Arrows indicate erosive pannus and inflammation.

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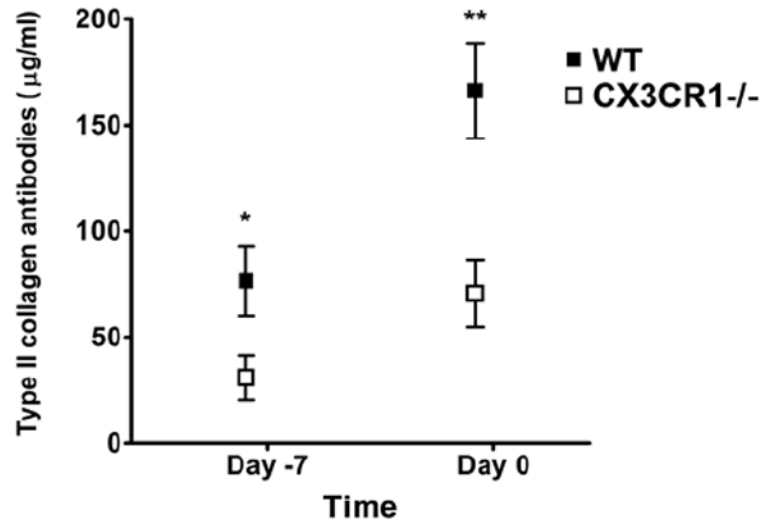

Figure 2. CX3CR1-/- mice have decreased autoantibody production compared to wildtype controls in CIA

Formation of anti-type II collagen autoantibodies was substantially decreased in CX3CR1-/- mice (n=13) compared to wildtype controls (n=13) at time points that preceded clinical disease (*p<0.05; **p<0.01), and this difference was more pronounced over time.

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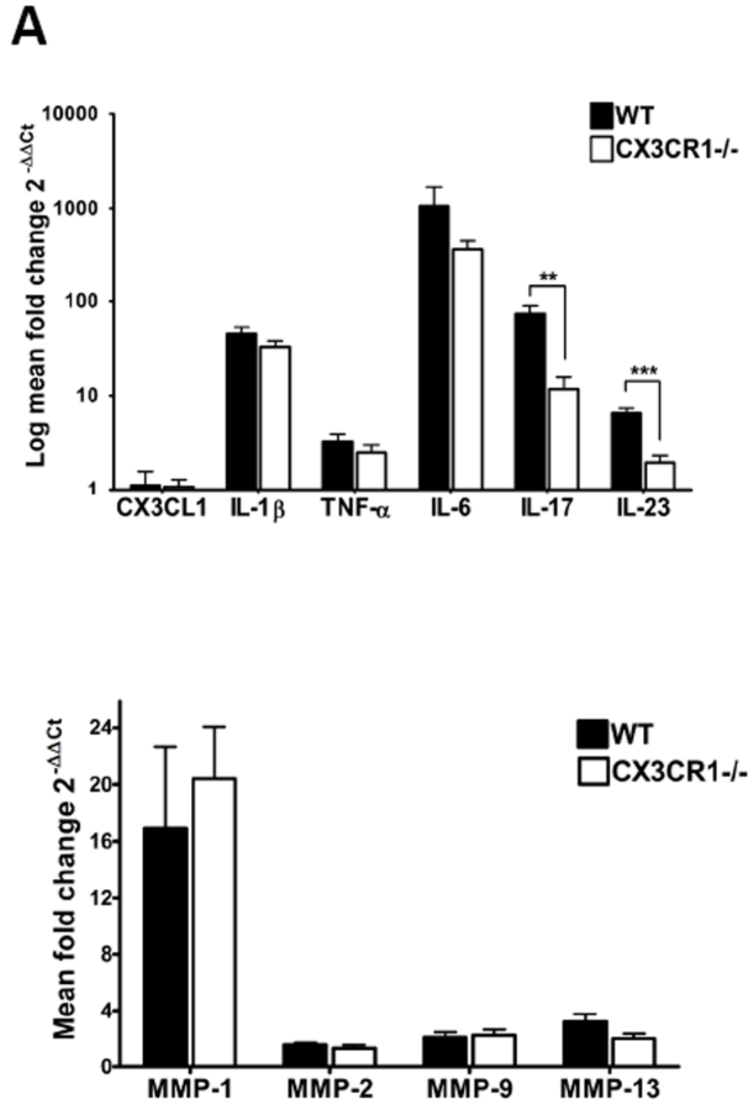

Figure 3. CX3CR1-/- mice have a statistically significant decrease in relative gene expression of IL-17 and IL-23 in paws with early inflammatory arthritis

Paws of both CX3CR1-/- (n=8) and wildtype (n=8) mice were collected at the earliest signs of clinical arthritis. Paws with substantial clinical disease (score=2) from both groups were collected and analyzed for **A**. proinflammatory cytokines and **B**. matrix metalloproteinases (MMPs) implicated in inflammatory arthritis. Only IL-17 (**p<0.01) and IL-23 (***p<0.001) showed a significantly reduced amount of relative gene expression in CX3CR1-/- mice compared to wildtype controls.

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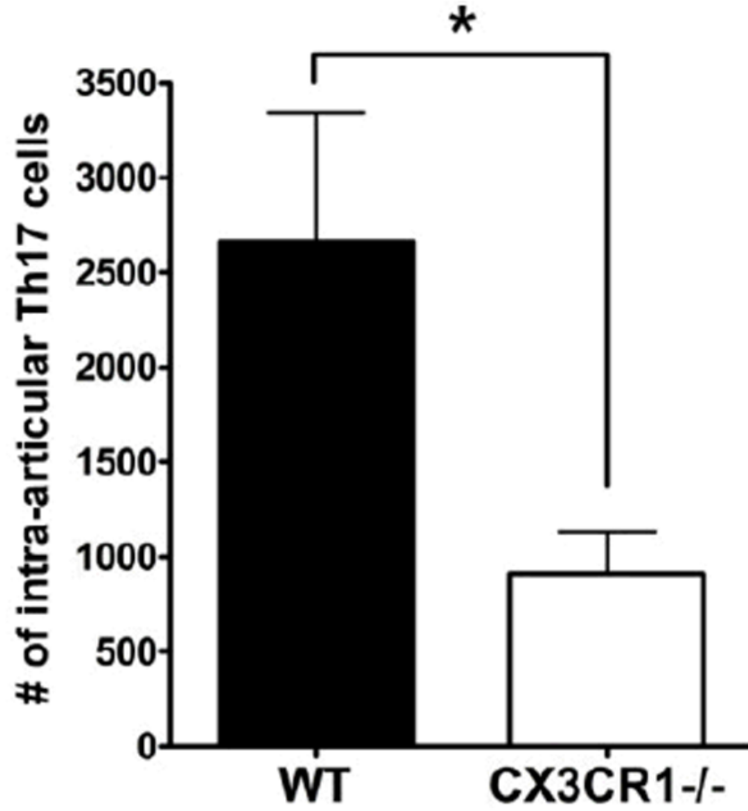

Figure 4. CX3CR1-/- mice have fewer Th17 cells in inflamed arthritic joints

The joint capsule of inflamed paws (score=2) from CX3CR1-/- (n=7) and wildtype (n=10) mice was dissected and dissolved with collagenase to liberate inflammatory cells, which were then stained to identify Th17 cells based on lymphocytes that had CD4+ surface expression and intracellular IL-17 by flow cytometry. Data are expressed as the mean absolute number of intra-articular Th17 cells per paw (*=$p<0.5$).

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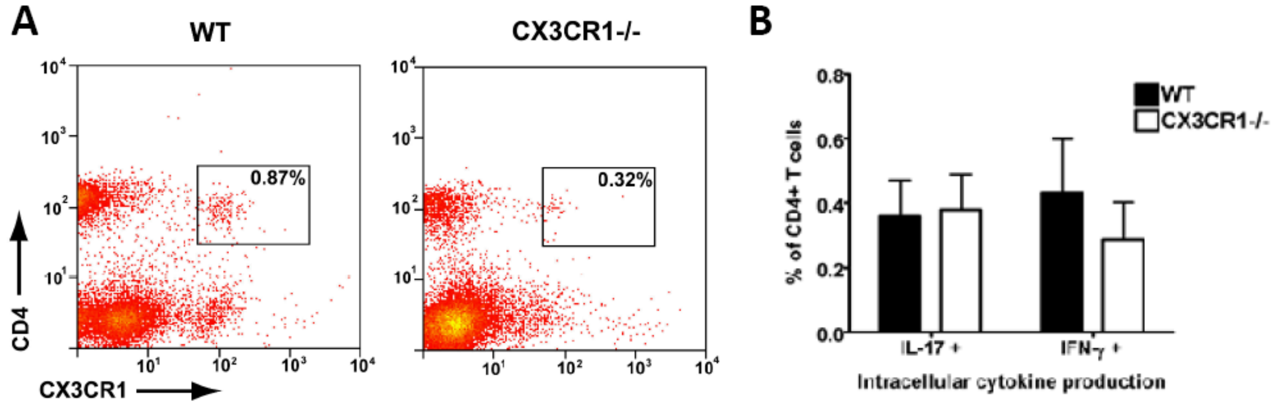

Figure 5. Wildtype CIA immunized mice have a subset of CD4+ T cells that express CX3CR1, but both CX3CR1-/- and wildtype stimulated CD4+ T cells are capable of producing Th1 and Th17 cytokines similarly

Draining lymph nodes (iliac and inguinal) were harvested from individual mice that had been immunized to develop CIA. **A.** Representative histogram of wildtype lymphocytes gated by forward and side scatter and then CD4+ CX3CR1+ surface expression. CX3CR1-/- lymphocytes were similarly gated as a staining control. **B**. Total lymphocytes isolated from draining lymph nodes were stimulated with anti-CD3 and anti-CD28 in culture, stained for surface CD4 and intracellular IL-17 or IFN-γ, and then analyzed by flow cytometry (CX3CR1-/-, n=5; wildtype, n=5).

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Table 1

T cell proliferative responses to CD3/CD28 engagement and type II collagen.

Genotype	Anti-CD3/CD28 (Mean cpm ± SEM)	Type II Collagen + APC (Mean cpm ± SEM)	Media (Mean cpm ± SEM)	CII % of Total Stimulated T cells ± SEM	Media % of Total Stimulated T cells ± SEM
WT	31850 ± 9178	533.4 ± 105.9	422.9 ± 78.91	2.695 ± 0.9413	2.286 ± 0.9152
CX3CR1-/-	28240 ± 5504	466.6 ± 26.34	447.0 ± 85.41	2.034 ± 0.3508	1.718 ± 0.2193