Experimental arthritis in CC chemokine receptor 2–null mice closely mimics severe human rheumatoid arthritis

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The prevailing paradigm is that in human rheumatoid arthritis (RA), the accumulation of monocytes and T cells in the joint, mediated in part by such CC chemokine receptors (CCRs) as CCR2 and CCR5, respectively, plays a central role in disease pathogenesis. To further validate this paradigm, we conducted proof-of-principle studies and tested the hypothesis that gene inactivation of *Ccr2* or *Ccr5* will ameliorate experimental RA. Contrary to our expectations, we found that in two well-established murine models of experimental RA, CCR2 expression in the hematopoietic cell compartment served as a negative regulator of autoantibody production as well as arthritic disease onset, severity, and resolution. In contrast, the RA phenotype in *Ccr5*-null mice was similar to that of WT mice. Remarkably, the collagen-induced arthritis phenotype of *Ccr2*$^{-/-}$ mice mimicked closely that of severe human RA, including production of rheumatoid factor, enhanced T cell production, and monocyte/macrophage accumulation in the joints. Our findings demonstrate an essential protective role of CCR2 expression in RA, indicate the existence of alternative receptors responsible for monocyte/macrophage accumulation to inflamed joints, and emphasize the need to clarify carefully the complex effects of the chemokine system in RA before they can be considered as therapeutic targets.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the infiltration of T and B cells, macrophages, and neutrophils into the synovial lining and fluid of the periarticular spaces. Chemokines act as ligands for highly related chemokine receptors that are expressed on distinct leukocyte cell populations, including the cell types recruited to the joint in RA (1). For example, the CC chemokine receptors (CCR) CCR5 (receptor for CCL5, CCL3, and CCL7) and CCR2 (receptor for CCL2, CCL12) are expressed on T cells and monocytes infiltrating the synovium of RA patients, respectively (2, 3). Supporting the role of chemokine receptors in RA pathogenesis, murine experimental arthritis can be partially ameliorated by blocking CCR5 and CCR2 using selective receptor antagonists (4–7). Thus, the prevailing paradigm is that the cellular influx to the inflamed joint in RA is choreographed through the temporal and spatially regulated expression of chemokines and their cognate receptors, prompting the hope of targeting specific members of the chemokine system for therapeutic purposes (1, 8). However, given the plethora of candidate chemokine–chemokine receptors medi-

ating cell recruitment pathways that can potentially contribute to the initiation and resolution of joint inflammation, it has been challenging to determine which receptors, if any, play unique, nonredundant roles in RA pathogenesis.

An especially informative means for evaluating the role of chemokine receptors in RA pathogenesis, including the initiation and resolution of joint inflammation, is the use of mice genetically inactivated for chemokine receptors. Collagen-induced arthritis (CIA) and collagen Ab–induced arthritis (CAIA) are two well-established murine models of human RA that have some distinct features (9, 10). In contrast to CIA, CAIA permits the analysis of both initiation and resolution of joint inflammation (11–15). In CIA, once Ab to type II collagen (CII) is formed, acute arthritis is triggered by Ab binding to collagen in the joints as well as neutrophil activation via Fc receptors, resulting in a disease process in which joint inflammation does not completely subside (9, 10, 14, 16, 17). In contrast, in CAIA the typical disease course involves a short period of acute disease followed by gradual resolution in joint inflammation (13). The CAIA model also permits an analysis of the contribution of the non-T and B cell compartment in the pathogenesis of experimental RA (12, 14, 15, 18–20).

To conduct proof-of-principle studies to elucidate the relative contribution of CCR5 and CCR2 in CIA and CAIA, we genetically inactivated these two receptors in an arthritis-prone murine strain, namely DBA/1J mice. Contrary to our expectations, genetic deletion of *Ccr2* was associated with markedly enhanced susceptibility to CIA and CAIA, whereas inactivation of *Ccr5* resulted in a CIA phenotype that was indistinguishable from that of WT mice. Our findings provide novel and unpredicted insights

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into the molecular determinants of RA, and suggest that distinct sets of chemokine receptors are likely to influence the induction and resolution phases of arthritis.

Methods

Mice. C57BL/6 and DBA/1J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). *Ccr2*- or *Ccr5*-KO mice were generated as described previously (21, 22). The KO strains were backcrossed to the inbred DBA/1J mice for either six (*Ccr5*$^{-/-}$ and *Ccr2*$^{-/-}$) or eight (*Ccr2*$^{-/-}$) generations. The KO and WT (+/+) mice resulting from mating of the final backcrossed heterozygotes (+/–) were used as breeders to generate the KO and control animals used in this study. Mice were bred and maintained under specific pathogen-free conditions. Some of the phenotypes observed in the *Ccr2*$^{-/-}$ mice — for example, increased Ab production following antigen (Ag) challenge (refs. 23–25 and this study), enhanced neutrophilic infiltration during inflammation (ref. 25 and this study), defective T cell apoptosis (ref. 26 and this study) — were observed in several disease models and/or diverse Ag challenges as well as in different mice strains (Supplemental Table 1; supplemental material available at http://www.jci.org/cgi/content/full/113/6/856/DC1). For this reason, to elucidate some of the potential mechanisms underlying the CIA phenotype observed in this study, for some selected experiments we used *Ccr2*$^{-/-}$ mice on a BALB/c or C57BL/6 background (> F$_6$ generation) with appropriate controls from Charles River Laboratories, Inc. (BALB/c; Wilmington, Massachusetts, USA) and The Jackson Laboratory (C57BL/6). The findings of these latter studies are described in Supplemental Tables 3–6 and 8–9. All experimental protocols adhered to IACUC guidelines and were approved by the institutional review boards at the University of Texas at Austin and the University of Texas Health Science Center at San Antonio, Texas, USA.

Induction and assessment of CIA. Arthritis induction and clinical follow-up for the experiments presented here were performed at the University of Texas at Austin, Texas, USA. Native bovine CII (Elastin Products Company, Inc., Owensville, Missouri, USA) was dissolved in 5 mM acetic acid with gentle stirring at 4°C for 4–8 hours. This solution was then emulsified with an equal volume of complete Freund's adjuvant containing 5 mg/ml heat-killed *Mycobacterium tuberculosis* (H37RA; Difco Laboratories, Detroit, Michigan, USA). Groups of 8- to 12-week-old WT, *Ccr2*$^{-/-}$, and *Ccr5*$^{-/-}$ male mice were immunized by intradermal injection at the base of the tail with 150 μg of CII. The mice were boosted 21 days later with an intradermal injection of 150 μg of CII in incomplete Freund's adjuvant. After the booster injection, mice were monitored two or three times per week for up to 130 days, and the onset and severity of disease were recorded. A clinical severity score assigned for each hindpaw and forepaw was based on the degree of swelling and redness as follows: grade 0, no swelling or erythema; grade 1, obvious swelling and redness in a single digit; grade 2, swelling and erythema in several digits; grade 3, severe swelling in a paw involving all the digits; grade 4, severe swelling with joint rigidity or deformity. The maximum possible clinical score per mouse was 16 (4 paws × 4 grades). To obtain the mean severity score (± SEM), this clinical score for each group of animals at the time point of evaluation was divided by the total number of arthritic mice.

Thioglycollate challenge. Intraperitoneal injection of thioglycollate, a well-established mouse model of acute peritonitis, was done as described (21). Peritoneal lavage was performed 72 hours after the intraperitoneal injection.

Harvesting of joint and lymph node leukocytes and FACS analysis. DMEM, RPMI 1640, medium 199, antibiotics, and FCS were purchased from Invitrogen Corp. (Carlsbad, California, USA). All chemicals were from Sigma-Aldrich (St. Louis, Missouri, USA), and Ab's were from Pharmingen (San Diego, California, USA), with the exception of the biotinylated Ab specific to receptor activator NF-κB ligand (RANKL), which was purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA). Cells infiltrating the joints were harvested by digesting the paws with collagenase for 2 hours at 37°C, and then rigorously homogenizing the tissue using glass slides. The debris was removed by using a 33% Percoll gradient, and the cells were counted and stained with fluorescent-labeled Ab's according to the manufacturer's instructions. Leukocytes were also prepared from the lymph nodes draining the inflamed joint. Draining lymph nodes (DLN) were homogenized by gentle crushing between the frosted ends of microscope slides. Erythrocytes were removed from spleen cell suspensions using a red-cell lysis buffer. The leukocytes were incubated with the indicated Ab's for 15 minutes at 4°C. The level of nonspecific staining was ascertained by using isotype-matched control Ab's. Cells were analyzed by flow cytometry on a FACScan using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Histochemical staining. The entire joint, together with the overlying skin tissue, was removed and fixed in paraformaldehyde (4% in PBS, pH 7.2), decalcified in 10% EDTA, and embedded in paraffin. Serial sections (5 μm) were made by cryostat sectioning and were stained. For tartrate-resistant acid phosphatase (TRAP) staining, the TRAP solution was prepared as follows: 9.6 mg of naphthol AS-BI phosphate substrate (Sigma-Aldrich) was dissolved in 0.6 ml of *N,N*-dimethylformamide (Sigma-Aldrich) with 60 ml of 0.2 M sodium acetate buffer (pH 5.0; Sigma-Aldrich) containing 84 mg of fast red-violet LB diazonium salt (Sigma-Aldrich), 58.2 mg of tartaric acid (Sigma-Aldrich), and 240 μl of 10% MgCl$_2$. The mixture was filtered through a 0.22-μm pore-size filter. Slides were incubated for 8 minutes in the staining solution at 37°C in the dark. The slides were then washed with water for 30 minutes and then subjected to counterstaining with methyl green for 5 minutes. Histological analyses was conducted by a pathologist who was blinded to the genetic differences in the experimental groups. Subsequently, a second investigator, also blinded to the study groups, reanalyzed the histopathological sections and took representative photomicrographs.

Radiological assessment of arthritis. Anteroposterior radiographs of the four limbs were obtained using a cabinet soft X-ray apparatus. An investigator blinded to the experimental groups examined the X-ray films.

Collagen-specific Ab's. Anti-CII Ab's were measured by ELISA as described previously (27). Briefly, the wells of 96-well round-bottomed plates were coated with CII (1–10 μg/ml). After blocking with 1% BSA, the wells were washed with PBS, and serially diluted sera from the mice immunized with CII was added and incubated overnight at 4°C. After washing, wells were incubated for 1 hour at room temperature with alkaline phosphatase–labeled anti-mouse IgG1 mAb's, anti-mouse IgG2a mAb's, or anti-mouse IgE mAb's. Wells were washed and were developed using *p*-nitrophenyl phosphate (pNPP), and ELISA readings were obtained as described previously (25, 27). Collagen-specific IgE mAb's were undetectable in these mice.

CAIA. Ccr2$^{-/-}$ mice and WT littermates (five mice per group) were injected intravenously with 4 mg of CII-specific mAb′s, a mixture of four mAb′s that recognize individual epitopes within the CB11 fragment of CII (Chemicon International, Temecula,

California, USA). After 48 hours had passed, mice received 25 µg LPS (Chemicon International) intraperitoneally and monitored for clinical signs of arthritis twice weekly for 3 weeks. The clinical score was similar to that for CIA.

Rheumatoid factor. For rheumatoid factor (RF) detection, ELISA plates were coated with purified mouse IgGs overnight at 4°C. All the subsequent steps were carried out at room temperature. Plates were washed to remove unbound IgG, blocked with 4% BSA for 1 hour, and washed again. Serum from immunized animals was added and incubated for 2 hours. After the plates were washed, biotin-labeled anti-mouse IgM mAb's were added and the plates incubated for 1 hour. After the plates were washed again, streptavidin–alkaline phosphatase (streptavidin-AKP) was added and the plates incubated for 1 hour. After a final wash, the plates were developed using pNPP (substrate for AKP) for 30 minutes and read in an ELISA plate reader.

Determination of Ab's to single-stranded DNA. The methods of Warren et al. were adopted (28). Briefly, methylated albumin–coated wells of a 96-well round-bottomed plate were coated with 10 µg/ml of denatured calf thymus DNA and incubated for 90–120 minutes (unless stated otherwise, all incubations were done at room temperature). After washing, the wells were blocked with BSA for at least 1 hour, after which the samples were added and incubated for 2–3 hours at room temperature or overnight at 4°C. Following this incubation, the plates were washed and anti-mouse Ig-AKP conjugated mAb's were added and the plates incubated for 1 hour. Finally, pNPP substrate was added; the plates were developed for 5–30 minutes and then read in an ELISA plate reader.

Bone marrow transfer. Recipient DBA/1J (WT or *Ccr2*−/−) mice or C57BL/6 WT mice received 1,000 cGy of irradiation from a cesium-137 source 8 hours prior to adoptive transfer of 5×10^6 whole bone marrow cells derived from the DBA/1J (WT or *Ccr2*−/−) or GFP+ C57BL/6 WT mice. In the GFP-transgenic strain, GFP is constitutively expressed in most cells including neural and bone marrow progenitor cells, and it was used as a marker for donor-derived cells. In the C57BL/6 recipients of bone marrow cells from GFP+ C57BL/6 mice, mouse cells were analyzed 10–12 weeks after cell transplantation; 90–95% of the recipient blood or spleen cells were GFP+, suggesting a high degree of chimerism in these adoptive transfer experiments (data not shown). Wild-type DBA/1J mice were lethally irradiated and transplanted with bone marrow cells from WT or *Ccr2*−/− DBA/1J mice. Additional controls included nontransplanted WT or nontransplanted *Ccr2*−/− mice. Three months after cell transplantation, the mice were immunized with collagen. Serum samples were collected 2 weeks after the second immunization, and anti-CII Ab levels were measured.

Cell labeling, apoptosis, and in vitro stimulation studies. To quantify and visualize the fractions of dividing T lymphocytes that were undergoing activation-induced cell death (AICD), we used the methods described by us previously (26) and outlined in detail in the Supplemental Notes.

Ribonuclease protection assay. Total RNA was isolated from the joints as described by Thornton et al. (29). Briefly, paws from WT or *Ccr2*-null mice were dissected and snap-frozen in liquid nitrogen, total RNA was then extracted. Ribonuclease protection assays (RPAs) were performed on the total RNA using the RiboQuant Multiprobe RNase Protection Assay Kit (Pharmingen). RNA from the inflamed or noninflamed paws from a given mouse was pooled for analysis. The data are presented as the ratio of the densitometric signals of a housekeeping gene (L32) and the mRNA for the gene of interest.

Ab's and cell culture for analysis of B cell responses. To study B cell responses in vitro, the methods of Reimold et al. were used (30). Briefly, mouse
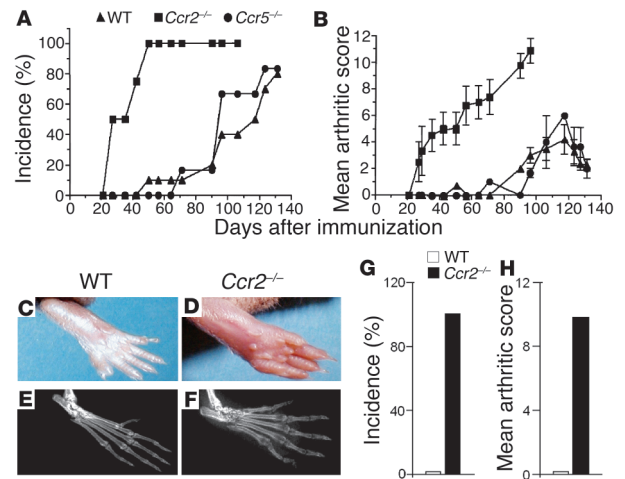

Figure 1
CIA incidence and severity is increased in *Ccr2*−/− mice. The data shown in this figure are representative of one of a minimum of three experiments. (**A–F**) In the experiment shown, *Ccr2*−/− mice (*n* = 8), *Ccr5*−/− mice (*n* = 6), and WT mice (*n* = 10) were given primary and booster injections of bovine CII and monitored two to three times per week for incidence and severity of arthritis. All mice were backcrossed six generations into the DBA/1J background. Days after immunization in **A** and **B** represent days after the second immunization with CII. (**A**) The cumulative number of arthritic animals in each group is shown as a percentage of the total number immunized with CII. (**B**) Arthritic score for each group at each time point was divided by the number of arthritic mice to calculate a mean severity score (± SEM). The maximal arthritic score for *Ccr2*−/− mice (11.0 ± 0.8) was significantly higher than the scores for WT (3.9 ± 0.7) and *Ccr5*−/− mice (3.2 ± 1.2) (*P* < 0.0001). The difference between WT and *Ccr5*−/− mice was not significant (*P* = 0.5). Photomicrographs (**C** and **D**) and radiographs (**E** and **F**) from WT and *Ccr2*−/− mice depicting severe arthritis and bone destruction and erosion in *Ccr2*−/− mice. (**G**) Incidence and (**H**) severity of arthritis determined 2 weeks after immunization with CII in F8 backcrossed *Ccr2*−/− (*n* = 3–4 mice per experiment) and WT mice (*n* = 3–4 mice per experiment).

splenocytes were plated at 10^6 cells/ml and stimulated for up to 4 days with anti-CD40 (1 µg/ml), anti-CD40 plus IL-4 (10 ng/ml), LPS (20 µg/ml) alone, or LPS (20 µg/ml) plus IL-4 (10 ng/ml). Subsequently, ELISAs for immunoglobulins (IgG1 and IgG2a) in the culture supernatants were performed. In some experiments, cell proliferation was determined by a nonradioactive cell proliferation assay that depends on the reduction by living cells of tetrazolium salt, MTT, to form a blue formazan product (Promega Corp., Madison, Wisconsin, USA).

Data collection and statistical analyses. The radiological, histopathological, and some immunological analyses described in this paper were performed 15–30 days after the second immunization with CII, a time point at which the arthritic disease peaked in *Ccr2*−/− mice, but was not clinically apparent in the WT mice. Data are expressed as the mean ± SEM. The two-tailed Student's *t* test for the difference between two means was used for comparing the maximal mean arthritic scores and for comparing anti-CII Ab levels in mouse sera. The Mann-Whitney test was used in instances when the data was not normally distributed.

Results

Increased incidence and accelerated joint destruction in Ccr2-deficient mice during CIA. In each of the five separate experiments performed, compared with WT or *Ccr5*−/− mice, *Ccr2*−/− mice consistently exhibited a

Table 1

Comparison of human RA and murine models of autoimmune arthritis

	K/BxN	MLR/lpr	*Ccr2*^{-/-}	Human RA
Clinical manifestations				
Arthritis	Symmetrical progressive erosive	Symmetrical progressive erosive	Symmetrical progressive erosive	Symmetrical progressive erosive
Etiology	Spontaneous	Spontaneous/induced	CII-induced	Multifactorial
Synovial thickening	Yes	Yes	Yes	Yes
Cartilage degeneration	Yes	Yes	Yes	Yes
Pannus formation	Yes	Yes	Yes	Yes
Immune manifestation				
RF	−	+	+	+
Cellular infiltrate in joints				
T cells	+	+/−[A]	++	++
B cells	+/−[A]	+/−[A]	+	+
Macrophages	++	++	+++	+++
Neutrophils	+	+/−[A]	+++	+
Cytokine profile				
IL-6	+	+	+	+
TNF-α	+	+	+	+
IFN-γ	?	?	+	+
Chemokine profile				
CXCL1 (MIP-2)	?	?	+	+
CCL2 (MCP-1)	?	+	+	+
CCL3 (MIP-1α)	?	?	+	+
CCL4 (MIP-1β)	?	?	-	+
Response to anti–TNF-α therapy	+/−[A]	Yes	?	Yes

[A]Conflicting results. An identical table with the addition of references appears as Supplemental Table 2. K/BxN is a mouse strain expressing the transgenic T cell receptor (which recognizes glucose–6-phosphatase isomerase) or a nonobese diabetic genetic background. MLR/lpr is a mouse strain that lacks the apoptotic gene called *FasL*.

significantly greater incidence and severity of CIA, whereas the CIA phenotypes of *Ccr5*^{-/-} and WT mice were nearly indistinguishable (Figure 1, A and B). *Ccr2*^{-/-} mice developed extensive swelling and severe ankylosis in multiple joints, whereas the disease in WT and *Ccr5*^{-/-} mice was usually restricted to single digits and rarely involved an entire paw (Figure 1, C and D). These findings were corroborated by radiological examination, which revealed involvement of multiple joints, substantial bone destruction, and decrease in bone density, all features characteristic of severe human RA (Figure 1, E and F). Since the lack of CCR5 expression did not modify significantly the course of CIA in DBA/1J mice, no further analyses were performed on the *Ccr5*^{-/-} animals.

To exclude any confounding that might occur as a result of the minimal genetic admixture in the F_6 *Ccr2*^{-/-} DBA/1J mice, we backcrossed these mice an additional two generations. Also, because in these experiments the F_6 WT littermates exhibited a benign course compared with *Ccr2*^{-/-} mice, we sought to determine whether further backcrossing of these littermates would alter their susceptibility to CIA relative to commercially available DBA/1J mice. Remarkably, compared with the F_6 backcrossed *Ccr2*^{-/-} mice, F_8 *Ccr2*^{-/-} mice were even more sensitive to the induction of CIA, and all the F_8 *Ccr2*^{-/-} mice developed arthritis within 14 days of the primary immunization (Figure 1G), whereas the F_6 *Ccr2*^{-/-} mice developed disease only after the booster injection with CII. Notably, at this early time point

the disease severity in the F_8 *Ccr2*^{-/-} mice was comparable to that observed 50 days after the booster immunization in the F_6 *Ccr2*^{-/-} mice (Figure 1H). Commercially available DBA/1J mice were only marginally more susceptible than the F_8 WT littermates, but the incidence and severity in these commercially obtained control animals remained significantly lower than that observed in the F_6 or F_8 *Ccr2*^{-/-} mice (data not shown).

In addition to the clinical and radiographic changes, by day 30 after the second immunization with CII, the *Ccr2*^{-/-} mice, but not the WT mice, had histopathological features that were highly reminiscent of severe human RA (Table 1 and Supplemental Table 2). The pathology in the joints of these KO mice was characterized by invasion of the pannus tissue into the bone space, destruction of the joint architecture, and severe erosion of cartilage surfaces accompanied by marked infiltration of inflammatory cells, primarily neutrophils and macrophages (Figure 2, A–F). In support of the notion that osteoclasts play an important part in the pathogenesis of focal bone erosion in arthritis (31–34), we observed enhanced osteoclast activity in the joints of *Ccr2*^{-/-} mice (Figure 2, C and D).

Several leukocyte cell subtypes contribute to human RA pathogenesis (Table 1 and Supplemental Table 2), including neutrophils. Although naive (unmanipulated) WT and *Ccr2*-null mice had similar numbers of circulating neutrophils, monocytes, and lymphocytes (Supplemental Table 3), after induction of CIA the joints of *Ccr2*^{-/-} mice had higher cell numbers than WT mice ($5.4 \pm 1.2 \times 10^6$ versus $2.9 \pm 1.0 \times 10^6$ cells; mean $\pm$ SD). FACS analyses revealed an increase in the percentage and total numbers of neutrophils and monocytes and/or macrophages recruited to the *Ccr2*-null mice (Figure 2, G and H, and data not shown). Mirroring this increased recruitment of monocytes, F4/80 transcripts, a marker characteristic for macrophages, were more abundant in the joints of *Ccr2*-null mice (Table 2). In addition, the abundance of transcripts characteristic for cell types such as B cells (CD19) and T cells (CD3, CD4, and CD8) were also higher in *Ccr2*-null mice than in WT mice (Table 2).

The enhanced recruitment of monocyte/macrophages to the joints of *Ccr2*^{-/-} mice was surprising in light of the extensive literature documenting a reduced recruitment of these cell types to inflammatory sites in different disease models tested in these KO mice (Supplemental Table 1). However, concordant with the findings in the literature, after intraperitoneal injection of thioglycollate in the naive DBA/1J mice, lower numbers of monocyte/macrophages were recruited to the peritoneum (Figure 2I). Thus, enhanced or reduced monocyte/macrophage recruitment following genetic inactivation of *Ccr2* in DBA/1J mice was clearly highly dependent on the nature of the inflammatory insult. Notably, analogous to our observations here, CCR2-independent recruitment of monocytes and macrophages has also been illustrated in a murine model of idiopathic pulmonary fibrosis (35).

Increased anti-CII Ab, RF, and anti–single-stranded DNA Ab production in Ccr2^{-/-} *mice.* CII-specific Ab's are necessary and sufficient to induce CIA (10, 16, 17), and thus enhanced anti-CII-specific Ab pro-
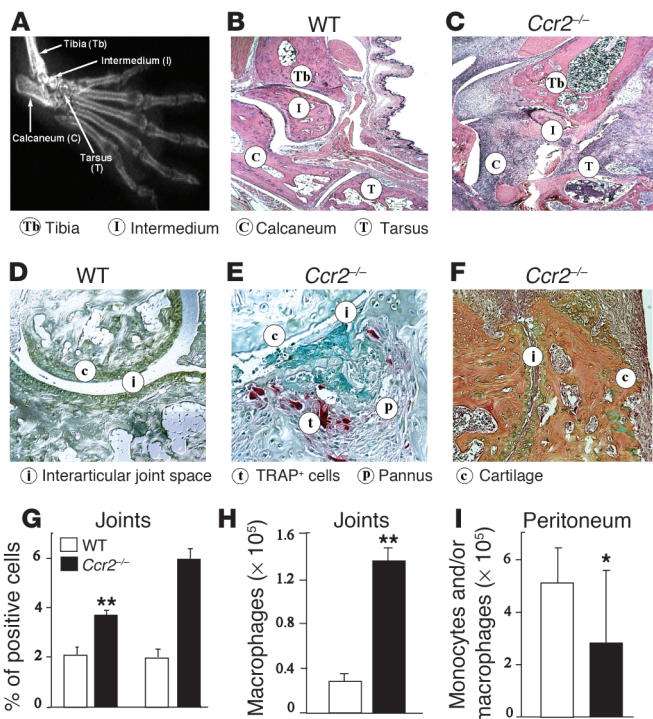
Figure 2

Histopathological and cellular responses in *Ccr2*−/− mice after induction of CIA or peritonitis. (**A**) X-ray of the paw of a *Ccr2*−/− mouse, shown to provide anatomical orientation. The abbreviated names of the bones highlighted in **B** and **C** are given in parentheses. Histopathological evaluation of the joints were conducted after (**B** and **C**) H&E, (**D** and **E**) TRAP (for osteoclasts), and (**F**) orange G (for enhanced bone visualization) staining. Affected joints derived from WT mice (**B** and **D**) show relatively normal articular surfaces, intact joint spaces, and absence of significant periarticular inflammation. *Ccr2*−/− mice (**C**, **E**, and **F**) showed irregular articular surfaces, collapsed joint space, loss of cartilage, and an increase in connective tissue adjacent to the joint space with abundant osteoclasts stained red by TRAP stain. Representative joints collected 30 days after the second immunization are shown. Note that, at this time point, there was little visual evidence of arthritis in the WT mice and hence the relatively normal histopathology in these mice (see Figure 1B). (**F** and **G**) To determine the nature of the cell types recruited, FACS analysis was performed on cells derived from the inflamed joints of four mice from each group. (**H**) Number of macrophages in inflamed joints. (**I**) Number of peritoneal monocytes/macrophages 72 hours after thioglycollate injection. *$P < 0.05$; **$P < 0.01$. Data shown are representative of one of three experiments. In **G**–**I**, the white and black histograms refer to WT and *Ccr2*-null mice, respectively.

duction may serve as a major pathogenic mechanisms underlying the severe CIA phenotype of *Ccr2*-null mice. Previous studies supported the notion that inactivation of *Ccr2* may be associated with increased Ag-specific Ab production. For example, we have previously shown that after *Leishmania major* infection, *Ccr2*-null mice in the C57BL/6 genetic background produce higher amounts of *L. major*–specific Ab's (25). Additionally, enhanced Ab production was also observed in a murine model of allergen hypersensitivity (refs. 23, 24 and Supplemental Table 1). Here, we extended these findings by showing that in the BALB/c background, inactivation of *Ccr2* was also associated with increased *L. major*–specific Ab's after intradermal infection (Supplemental Table 4).

We further extend the notion that inactivation of *Ccr2* is associated with aberrant Ab responses to different immunogenic challenges and across different genetic backgrounds, since *Ccr2*−/− DBA/1J mice produced significantly higher levels of anti-CII IgG2a and anti-CII IgG1 compared with WT mice 2 weeks after the primary immunization (Figure 3, A and B). We selected this early time point for analyses of Ab production for two reasons. First, since there is a strong correlation between anti-CII Ab titers before the onset of arthritis and subsequent arthritic severity (36), we surmised that elevated levels of anti-CII Ab at this early time point would support a possible link between aberrant Ab production and the increased disease severity observed in *Ccr2*-null mice. Second, this early time point we posited would potentially limit the confounding that might occur once clinical arthritis is evident, since the disease per se may act as a feedback mechanism to increase autoantibody production, and therefore the finding of abnormal Ab production during the later stages of disease may not necessarily represent a primary immune abnormality. In this light, our findings suggest that CCR2-dependent processes are associated with the magnitude of anti-CII Ab production, and may be associated with the more severe CIA phenotype observed in *Ccr2*-null mice. However, we cannot exclude the possi-

bility that clinically undetectable joint disease during the very early phases of CIA also contributed to the enhanced anti-CII Ab production in *Ccr2*−/− null mice (37).

We next determined whether the production of other autoantibodies was also increased after CII immunization in *Ccr2*−/− mice. Since among all biomarkers currently known for human RA, autoantibodies against IgG (e.g., RF) have consistently been shown to be the best predictor of disease severity (38–40), we measured RF 2 weeks after primary immunization in WT and *Ccr2*−/− mice. After induction of CIA, RF was undetectable in serum from WT mice, a finding concordant with previous studies (10, 17, 39, 41, 42). However, RF was detected in the sera of *Ccr2*−/− mice (Figure 3C), thus reproducing one of the characteristic features of human RA, and notably, a feature of only a handful of other rodent models of RA (Table 1, and refs. 38–40, 42–46). Furthermore, compared with WT mice, higher levels of circulating anti–single-stranded DNA (anti–

Table 2

Cellular phenotype in joints derived from CII-immunized mice

mRNA	WT	*Ccr2*−/−	P
CD3ε	0.12 ± 0.01	0.16 ± 0.03	0.017
CD4	0.13 ± 0.01	0.19 ± 0.03	0.006
CD8α	0.17 ± 0.02	0.24 ± 0.05	0.028
CD8β	0.16 ± 0.03	0.23 ± 0.05	0.031
CD19	0.18 ± 0.04	0.25 ± 0.03	0.024
F4/80	0.43 ± 0.07	0.57 ± 0.06	0.011
CD45	0.43 ± 0.07	0.56 ± 0.05	0.012

RPA was performed on joints derived from WT or *Ccr2*-null mice 25 days after the second CII immunization. Each value represents the ratio between the densitometry reading of the gene of interest and the housekeeping gene L32, and the data are presented as mean ± SD (4–5 joints pooled per sample; total of 20 joints).
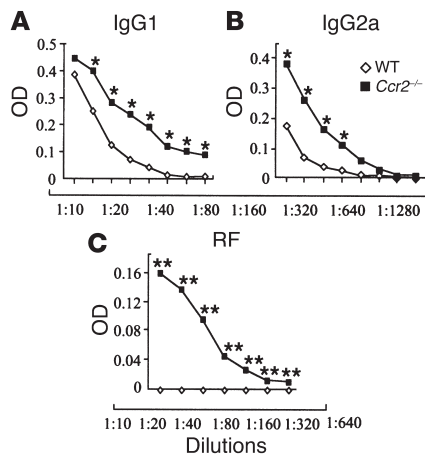
Figure 3
Elevated levels of autoantibodies 2 weeks after CII immunization in *Ccr2⁻/⁻* mice, as shown by OD measurements in serially diluted serum samples. (**A**) IgG1 and (**B**) IgG2a against collagen (mean of three WT and four *Ccr2*-deficient mice); *$P < 0.05$. (**C**) RF was measured in the sera of three WT and four *Ccr2⁻/⁻* mice; **$P < 0.0001$. Collagen-specific IgG quantification was performed in several experiments, and *Ccr2⁻/⁻* mice consistently had higher IgG levels than the WT littermates before arthritic disease was clinically evident, and those levels remained elevated after the arthritic disease started (data not shown).

ssDNA) Ab's were detected in the *Ccr2⁻/⁻* mice (in WT mice, OD = 0.049 versus *Ccr2*-null mice, OD = 0.165; $P = 0.001$).

Given that an aberrant production of autoantibodies, especially anti-CII Ab's, appeared to be a major feature of the severe CIA phenotype in *Ccr2⁻/⁻* mice, we next tested the hypothesis that this was secondary to an intrinsic defect(s) at the level of *Ccr2*-null B cells that was present in these mice before CII immunization (e.g., enhanced proliferation, increased Ab production, accelerated maturation). Several lines of evidence suggested that *Ccr2*-null B cells before induction of CIA were normal. First, in vitro experiments showed that after stimulation of splenocytes with anti-CD40 (1 μg/ml), anti-CD40 plus IL-4 (10 ng/ml), LPS (20 μg/ml), or LPS (20 μg/ml) plus IL-4 (10 ng/ml), DBA/1J WT and *Ccr2⁻/⁻* mice splenocytes produced similar amounts of Ab (data not shown) and had similar proliferation rates (Supplemental Table 5). Second, following B cell receptor stimulation using IgM F(ab′)2 fragments, mature B cells derived from the spleens of WT and *Ccr2⁻/⁻* mice appeared to have comparable phosphorylation levels of tyrosine kinases, including that of Syk, a critical proximal kinase that initiates all B cell receptor–dependent signaling pathways (47) (Supplemental Notes and data not shown). Third, the percentage of pro- (B220⁺CD43⁺IgM⁻), pre- (B220⁺CD43⁻IgM⁻), immature (B220⁺IgM⁺IgD⁻), and mature (IgM⁺IgD⁺B220hi) B cell populations in the spleens, bone marrow, and peritoneal cavities of naive (nonimmunized) WT and *Ccr2⁻/⁻* mice was similar, as were B-1 cells (B220⁺IgM⁺CD5⁺) (Supplemental Table 6). Taken together, these findings suggest that B cell responses in vitro as well as the B cell development in *Ccr2*-null mice do not differ significantly from WT mice.

Since we found no gross defects at the level of *Ccr2⁻/⁻* B cells prior to CII immunization to account for the severe CIA phenotype, we next determined the contribution of T cell–mediated immune responses to enhanced Ab production and joint inflammation: cytokine production at the local (joint) and the secondary

lymphoid organ level and intrinsic abnormalities in the *Ccr2*-null T cells (i.e., Th cell polarization and AICD).

Cytokine responses in Ccr2⁻/⁻ mice following CII immunization. There is considerable evidence to suggest that in DBA/1J mice, CIA is a Th1-mediated inflammatory disease (5, 10, 48, 49). Typically, Th1 responses are evaluated by determining cytokine levels from the spleen or DLN of mice with CIA. However, recent studies indicate that the cytokine responses in the peripheral lymphoid organs reflect the immune reactivity against the mycobacterial component of the adjuvant, whereas the cytokine responses in the joints may mirror more accurately the immune response against collagen and not the adjuvant (9, 50, 51). Additionally, we reasoned that although several cytokines could be upregulated systemically, elucidation of those cytokines that drive the inflammatory process at the level of the joints (i.e., local microenvironment) may be especially informative regarding the Th mechanisms underlying the severe joint inflammation that we observed in *Ccr2*-null mice. For these reasons, we first determined the cytokine levels by RPA in the inflamed and noninflamed joints of *Ccr2⁻/⁻* mice.

Consistent with the notion that high levels of IFN-γ (9) may be a pathogenic feature of severe joint destruction in CIA, there was abundant expression of this cytokine in the inflamed, but not the noninflamed joints of *Ccr2⁻/⁻* mice derived from the same animal, whereas in contrast, Th2 cytokines (i.e., IL-4, IL-5, and IL-13) were undetectable (Table 3). Furthermore, the expression of CCL3, a chemokine associated with Th1 responses (52), as well as other proinflammatory chemokines, were also upregulated in the inflamed joints of *Ccr2*-null mice (Table 3). Notably, in light of the increased recruitment of neutrophils to the *Ccr2*-null joints, the expression of macrophage inflammatory protein 2 (MIP-2), a chemokine that is fairly specific for recruitment of neutrophils, was also elevated (53–55).

Table 3

Inflammatory mediators in joints derived from CII-immunized *Ccr2⁻/⁻* mice

	Noninflamed	Inflamed	*P*
Th1 cytokines			
IFN-γ	ND	0.54 ± 0.06	0.00001
IL-2	ND	ND	–
Th2 cytokines			
IL-4	ND	ND	–
IL-5	ND	ND	–
IL-10	ND	ND	–
IL-13	ND	ND	–
Proinflammatory cytokines			
IL-6	0.28 ± 0.04	0.69 ± 0.11	0.02
IL-9	ND	ND	–
IL-15	0.20 ± 0.01	0.24 ± 0.05	0.14
Chemokines			
CCL1 (TCA-3)	ND	ND	–
CCL2 (MCP-1)	0.40 ± 0.02	0.75 ± 0.07	0.01
CCL3 (MIP-1α)	ND	0.32 ± 0.03	0.0001
CCL4 (MIP-1β)	ND	ND	–
CCL5 (RANTES)	ND	ND	–
CXCL1 (MIP-2)	ND	0.43 ± 0.02	0.0002
CXCL10 (IP-10)	ND	ND	–

RPA was performed on the inflamed and noninflamed joints of *Ccr2⁻/⁻* mice 25 days after the second CII immunization. Each value represents the ratio between the densitometry reading of the gene of interest and L32, and the data are presented as mean ± SD (4–5 joints pooled per sample; total of 20 joints). ND, not detected.

Table 4

Inflammatory mediators in joints derived from CII-immunized mice

mRNA	WT	*Ccr2*−/−	*P*
TNF-α	ND	ND	–
IL-6	0.28 ± 0.05	0.67 ± 0.10	0.001
TNFRp55	0.54 ± 0.12	0.65 ± 0.16	0.182
TNFRp75	ND	ND	–
CCR1	0.11 ± 0	0.29 ± 0.06	0.002
CCR2	ND	ND	–
CCR5	0.22 ± 0.01	0.34 ± 0.05	0.009
CCR7	ND	ND	–

RPA was performed on joints derived from WT or *Ccr2*-null mice 25 days after the second CII immunization. Each value represents the ratio between the densitometry reading of the gene of interest and L32, and the data are presented as mean ± SD (4–5 joints pooled per sample; total of 20 joints). ND, not detected.

In addition to these Th1/Th2 cytokines, we determined the expression profile of additional inflammatory mediators such as IL-6 and TNF-α and chemokine receptors in the joints of WT and *Ccr2*-null mice (Table 4). Notably, an enhanced expression of IL-6, CCR1, and CCR5 was detected in *Ccr2*-null mice, but there were no differences in the expression levels of TNF-α or its receptors p55 and p75 between KO and WT mice (Table 4). These findings, taken together with the data shown in Table 3, suggest that a biased localized Th1 response and increased IL-6 production in *Ccr2*−/− mice may play a significant role in driving the inflammatory processes in the joint, and that the enhanced expression of several chemokines and their cognate receptors may account for the nature of the cellular infiltrate in the inflamed joints. At least at this early time point, the TNF system did not appear to contribute significantly to joint pathology; however, it is conceivable that at other time points during CIA this gene system may also play a key role.

To extend the aforementioned studies, we also determined the spontaneous as well as Ag (CII)-specific IFN-γ production from the spleens and DLN of WT and *Ccr2*-null mice 25 days after the second immunization, a time point at which joint inflammation was evident in the KO mice. Spontaneous IFN-γ (mean ± SD) production in the DLN (498 ± 284 pg/ml) and spleens (25 ± 33 pg/ml) was evident only in the *Ccr2*-null mice. Additionally, the Ag-induced IFN-γ levels in the DLN were also higher in *Ccr2*−/− (3,500 ± 147 pg/ml) compared with WT (1.7 ± 3 pg/ml; *P* < 0.001) mice. Ag-induced IFN-γ production in the spleens of *Ccr2*−/− (1,830 ± 803 pg/ml) and WT (1,174 ± 1,890 pg/ml) mice was similar. Interestingly, although IL-4 expression was not detected in the inflamed joints of *Ccr2*-null mice, the Ag-specific IL-4 production from the spleens (WT: 12 ± 12 pg/ml; *Ccr2*−/−: 182 ± 81 pg/ml; *P* < 0.05) and the DLN (WT: 2.3 ± 1.3 pg/ml; *Ccr2*−/−: 66 ± 64 pg/ml; *P* < 0.08) was also increased in *Ccr2*-null mice compared with WT mice. Thus, in the inflamed joints a Th1 response may be a pathogenic variable leading to joint destruction, whereas the systemic Th0 responses may reflect the responses to collagen as well as *Mycobacterium* Ag's (9).

One possibility to account for this Th1-bias in the relevant local microenvironment was that this Th1 bias was secondary to the genetic background — that is, it was specific to the DBA/1J background in which CIA was elaborated. An alternative possibility is that this Th1 bias is due to the specific nature of the immunogen. To differentiate between these two possibilities, we resorted to the well-characterized model system used to characterize Th1/Th2 polarization in vivo, that is, the infection model with *L. major*. DBA/1J mice are normally resistant to *L. major* infection (56); however, we found that after intradermal infection of the ears of WT and *Ccr2*-null mice with *L. major*, these *Ccr2*-null mice in a DBA/1J background were highly susceptible to infection (increased ear swelling, higher parasite burdens) and increased Th2 (IL-4) levels in the DLN (Supplemental Table 7, and data not shown). Notably, susceptibility to *L. major* is characteristically associated with an increase in Th2 cytokines. Thus, it would appear that the Th response in DBA/1J mice is secondary to the nature of the immunological challenge: in CIA it is associated with a Th1 response, whereas in *L. major* infection it is associated with a Th2 response. Further supporting the notion that *Ccr2*-null mice can develop a Th1-biased response, we found that in a graft-versus-host-disease (GVHD) model, *Ccr2*-null mice in a C57BL/6 background develop an enhanced Th1 response associated with a poor GVHD outcome (26).
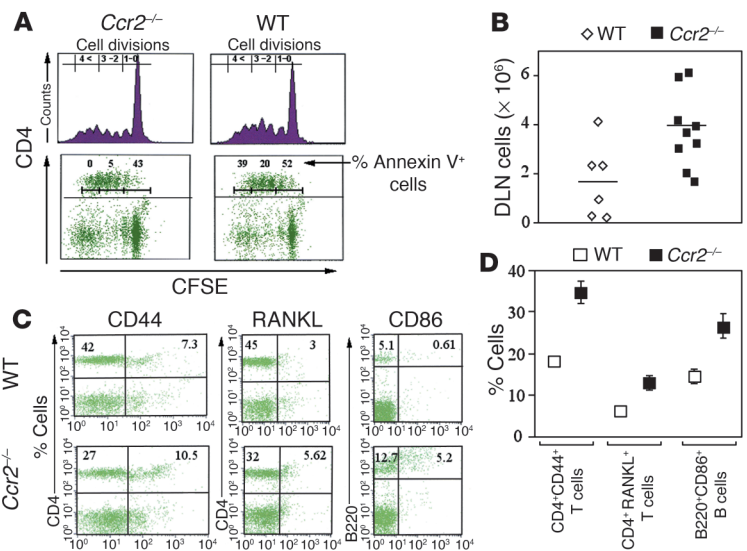

Figure 4

Higher accumulation of activated T and B cells in vivo, and decreased AICD in vitro in *Ccr2*−/− mice. (**A**) Quantitative analysis of proliferation and apoptosis of WT or *Ccr2*-null splenocytes derived from naive mice after stimulation with anti-CD3. carboxy-fluorescein succinimidyl ester–labeled (CFSE-labeled) splenocytes from WT and *Ccr2*−/− mice were stimulated with soluble anti-CD3 antibody (2 μg/ml), and cellular proliferation was assessed by FACS after 72 hours. CFSE-labeled cells were also stained for CD4 and Annexin V, and the percentage of CD4+AnnexinV+ cells in each cell division was determined. The number of generations is noted in the upper left of each panel. Data shown in this panel are representative of at least three separate experiments derived from two animals per group. Single-cell suspensions were prepared from arthritic joints DLN derived from WT or *Ccr2*−/− mice 30 days after the second immunization with CII. Cell numbers (**B**) and activation markers on T and B cells (**C** and **D**) in the DLN of WT and *Ccr2*−/− mice are depicted. The cells were counted and stained with the fluorescent-labeled Ab's shown in **B** and **C**. In **D**, the percentage of activated T cells (CD4+/CD44+) or (CD4+/RANKL+) or B cells (B220+/CD86+) within the total T or B cell population is shown. The findings shown in **A**–**C** are representative of three separate experiments, and the data depicted are from three WT and five *Ccr2*−/− mice.

Table 5

Transfer of CIA phenotype to WT mice by hematopoietic cell transplant.

| Mice | | Incidence | Mean arthritic |
Donor	Recipient		score
WT →	WT	0/5	0
Ccr2⁻/⁻ →	WT	4/5	9.6
WT		0/5	0
Ccr2⁻/⁻		4/5	9.6

Normal Th polarization and proliferation but decreased AICD in Ccr2⁻/⁻ T cells. We next determined whether abnormalities intrinsic to *Ccr2*-null T cells that are present before CII immunization could provide insights into the mechanisms leading to a severe CIA phenotype. In light of the observation that there was increased IFN-γ cytokine production in the inflamed joints as well as the DLN of *Ccr2*-null mice, we determined whether *Ccr2*-null Th cells had a higher intrinsic ability to produce Th1 cytokines after T cell receptor stimulation and under Th1-polarizing conditions. We found that highly purified naive T cells derived from WT and *Ccr2⁻/⁻* mice were comparable in their ability to differentiate and produce canonical Th1 or Th2 cytokines under appropriate polarizing conditions (Supplemental Table 8).

In light of the findings of normal Th1 and Th2 skewing in vitro, we hypothesized that either abnormal proliferation or T cell AICD rates in naive (unchallenged) *Ccr2⁻/⁻* mice may contribute to both increased cytokine and T cell–dependent Ab production. Although WT and *Ccr2⁻/⁻* DBA/1J mice had similar rates of T cell divisions, *Ccr2*-null splenocytes had a significant reduction in the percentage of apoptotic T cells after stimulation with anti-CD3 (Figure 4A). A similar phenotype was recapitulated when *Ccr2⁻/⁻* or WT T cells were stimulated with anti-CD3 plus anti-CD28. After 72 hours of stimulation with anti-CD3/CD28, the percentage of CD4⁺ *Ccr2*-null apoptotic cells was significantly lower than in WT T cells (data not shown). These findings are concordant with our recent observation that *Ccr2*-null T cells derived from KO mice on a C57BL/6 background also had impaired AICD (26). This defect in AICD did not appear to be secondary to a gross defect in T cell receptor–dependent signaling, since the tyrosine phosphorylation patterns of critical kinases such as PLC-γ–1, ZAP-70, and MAPK1/2 induced by T cell receptor cross-linking using anti-CD3 Ab in highly purified T cells derived from WT and *Ccr2⁻/⁻* mice was similar (Supplemental Notes and data not shown).

If the aforementioned in vitro findings of reduced AICD in *Ccr2*-null T cells were potentially contributing to the CIA phenotype in *Ccr2*-null mice, we surmised that we should observe an increase in the accumulation of activated T and B cells after induction of CIA in *Ccr2⁻/⁻* mice and be able to transfer the disease phenotype to WT animals by adoptive transfer of *Ccr2*-null whole bone marrow cells and/or purified T cells.

Potential contributions of reduced AICD in Ccr2⁻/⁻ mice to the CIA phenotype: accumulation of activated T and B cells. CII-immunized *Ccr2⁻/⁻* mice exhibited prominent lymphadenopathy. Consistent with this observation, the DLN of *Ccr2*-null mice had a greater cellular content (Figure 4B) that was associated with increased total numbers and percentages of activated T cells (CD4⁺CD44⁺ cells; Figure 4, C and D). Notably, we also found that the lymph nodes of *Ccr2⁻/⁻* mice had a

higher accumulation of T cells that expressed the osteoclastogenic factor RANKL (CD4⁺RANKL⁺) that might potentially account for the severe bone loss observed in these mice (Figures 1F and 4, C and D) (32, 57). In addition, the DLN of *Ccr2⁻/⁻* mice had nearly three times as many B cells and, more importantly, the number of activated B cells was four times higher in these mice compared with WT mice (Figure 4, C and D, and data not shown). Thus, the increased cellularity in the DLN of *Ccr2⁻/⁻* mice was accompanied by an enhanced activation state of the T and B cells. This accumulation in the number of activated T cells was observed only after CII immunization, since prior to CIA induction the percentage of naive as well as activated and memory T cells in *Ccr2⁻/⁻* and WT mice was comparable (Supplemental Table 9).

Potential contributions of reduced AICD in Ccr2⁻/⁻ mice to the CIA phenotype: adoptive-transfer studies. Bone marrow cells from naive WT or *Ccr2⁻/⁻* mice were adoptively transferred into lethally irradiated WT mice. At 3 months after transplant, a time point at which the hematopoietic system of the recipients was reconstituted (95% chimerism as described in Methods), we immunized these mice with CII. Wild-type mice reconstituted with WT bone marrow cells did not develop arthritis 3 months after the second immunization with CII (Table 5). In contrast, WT mice transplanted with *Ccr2⁻/⁻* bone marrow cells developed frank arthritis within 30 days after the second immunization with clinical arthritic scores similar to those observed in untransplanted *Ccr2⁻/⁻* mice immunized with CII (Table 5). In addition, compared with WT mice that were recipients of WT bone marrow cells, higher levels of anti-collagen IgGs were detected in WT recipients of *Ccr2⁻/⁻* bone marrow cells (Figure 5). Taken together, these findings indicated that WT DBA/1J mice adoptively transferred with *Ccr2⁻/⁻* cells had a CIA phenotype that mimics closely that observed in untransplanted *Ccr2⁻/⁻* DBA/1J mice, demonstrating that the expression of CCR2 in the hematopoietic compartment is a critical determinant of CIA.

Whether the CIA phenotype can be adoptively transferred readily from CII-immunized donor mice into a syngeneic host (not allogeneic SCID) by transplantation of only purified T cells is not clear, since only very few studies have demonstrated that this is possible
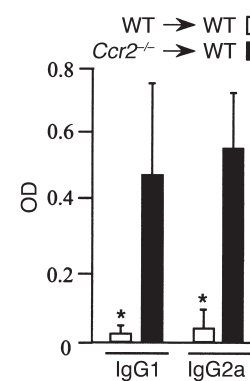

Figure 5

Transplantation of CCR2-null hematopoietic cells is sufficient to transfer the CIA phenotype to WT animals. Bone marrow cells derived from WT and *Ccr2⁻/⁻* mice were transplanted into WT recipients that had been lethally irradiated (1,000 cGy). Three months after transplantation, the protocol to induce CIA was initiated as described in the Methods section. Collagen-specific Ab's determined in the serum of transplanted animals two weeks after the second immunization.
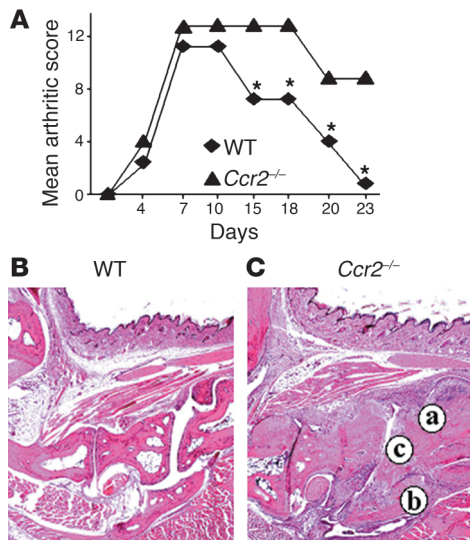
Figure 6
Enhanced CAIA phenotype in *Ccr2*⁻/⁻ mice. (**A**) Mean arthritic score after induction of CAIA was determined in WT and *Ccr2*⁻/⁻ mice. Histopathological analyses (H&E staining) of joints from (**B**) WT and (**C**) *Ccr2*⁻/⁻ mice. Significant joint destruction was evident in *Ccr2*⁻/⁻ mice compared with WT mice. Letters indicate (a) chronic inflammatory infiltrate and extensive synoviocyte proliferation; (b) loss of interarticular space; (c) cyst formation. *$P < 0.001$.

(58). Most adoptive-transfer studies have used CII-specific T cell clones or whole splenocytes followed by reimmunization of the mice with CII to induce disease (59–63). Nevertheless, we attempted to transfer the CIA phenotype by adoptive transfer of purified T cells using the protocols of Delgado et al. (58). WT or *Ccr2*-null DBA/1J mice were immunized with CII, and 14 days after immunization, T cells purified from their spleens were transferred intravenously into naive DBA/1J mice; the recipient mice were not subsequently challenged with CII. Up to 100 days after transplant we observed no phenotypic changes in the recipient mice.

Ccr2-null mice are highly susceptible to CAIA. The combined injection of anti-CII Ab's on day 0 and LPS on day 2 induced arthritis in both WT and *Ccr2*⁻/⁻ mice (Figure 6A). The first sign of slight swelling was detected around day 4, and paw swelling with redness reached a maximum on days 6–7. As demonstrated in previously published reports, we found that the CAIA-affected animals developed swollen, red, and ankylosed joints (12, 20). The swelling and associated other arthritic changes continued until day 10–12 in WT and *Ccr2*⁻/⁻ mice. However, after this time point we observed a differential response in the resolution phase of the disease: signs of arthritis gradually decreased after day 12 and were minimal by day 23 in WT mice, whereas in *Ccr2*⁻/⁻ mice signs of severe arthritis persisted, suggesting a delay in the processes that lead to a resolution in inflammation. Histological analysis confirmed these findings, since 23 days after injection of the arthritogenic Ab's the joints of the WT mice had minimal signs of disease, whereas the joints of *Ccr2*⁻/⁻ mice had prominent signs of chronic arthritis with pannus formation, and destructive bone and cartilage erosion, predominantly of the distal joints (Figure 6, B and C). Collectively, these findings indicated a possible requirement of CCR2 expression for the resolution of the ongoing inflammatory processes during CAIA.

Discussion

Although animal models may not reproduce all the features of human RA, they can help understand normal inflammatory and immune responses during RA pathogenesis or serve as vehicles to test novel therapeutic agents. The information obtained from these models and human studies informs the current view of RA pathogenesis in which an initial T cell–dependent phase, characterized by the development of T cell Ag-specific autoreactivity leading to the generation of autoantibodies, is followed by a T cell–independent phase characterized by Ab deposition, complement activation, leukocyte infiltration into the synovium, and tissue destruction (9, 10, 13, 16). Within this conceptual framework, the findings obtained in the *Ccr2*-null mice after induction of CIA suggest that, contrary to our expectations, chemokine-dependent processes may play important roles during this T cell–dependent (e.g., AICD and production of autoantibodies) and T cell–independent phases of RA pathogenesis (e.g., leukocyte infiltration).

Furthermore, our results also suggest the intriguing possibility that in CIA either CCR2 is dispensable for the migration of monocyte/macrophages into inflamed joints, or CCR2⁻ subpopulations of monocyte/macrophages accumulate in inflamed joints and contribute to disease pathogenesis. The existence of monocyte populations that can be subdivided based on their CCR2 expression (positive and negative) levels has recently been demonstrated by elegant studies from the Littman lab (64). Although these authors suggest that the CCR2⁻ monocyte cell populations may migrate only to the noninflamed sites to become resident macrophages, it is conceivable that in the context of specific disease processes and cellular compartments such as CIA, or even human RA, this CCR2⁻ leukocyte population may infiltrate into the inflamed joints. For example, although 84% of circulating monocytes in normal subjects expressed CCR2, only 76% of monocytes expressed CCR2 in patients with RA, but more importantly, even fewer monocytes in the synovial fluid from RA patients expressed CCR2 (24%), suggesting that the majority of the monocytes recruited to RA joints may be CCR2⁻ (65). This reduced detection of CCR2⁻ monocytes was thought not to be secondary to ligand-mediated downregulation of the receptor, since the expression of other chemokine receptors such as CCR5 on this cell population in the synovial fluid (47%) was higher than in the peripheral circulation (17%) (65).

Another important feature of the CIA phenotype observed in *Ccr2*-null mice was the presence of RF. Even though the sensitivity and specificity of RF for identification of patients with RA is relatively low compared with the more recently discovered autoantibodies, several studies show that RF predisposes to the development of RA (38, 43, 44). Increased levels of RF in symptom-free individuals greatly increase the risk of acquiring RA, and high titers predict persistent radiographic damage in inflammatory polyarthritis (38, 43, 44). Moreover, RF levels are also elevated in relatives of RA patients and RA-prone rats, providing evidence of an independent genetic control of RF production (38, 43, 44). Thus, identification of the mechanisms that promote RF production specifically, and enhanced Ab production in general, in *Ccr2*-null mice could be helpful in understanding the pathogenesis of RA.

Ccr2-null mice are one of the most intensely scrutinized chemokine receptor KOs studied to date (Supplemental Table 1). Diverse phenotypes in these mice have been described after infectious, inflammatory, autoimmune, transplant, and other challenges. Ascribing a precise mechanism that is applicable to each of these phenotypes or common mechanistic features has been a daunting

task. Initial studies suggested that a prominent Th2 skewing and/or impaired monocyte/macrophage migration was a common denominator for several of the phenotypes observed in mice genetically inactivated for *Ccr2*. However, these two characteristics are not universal features of the phenotype of *Ccr2*-null mice in the diverse disease models examined (Supplemental Table 1). Thus, the role of CCR2 in the immune response appears to be more variable than initially anticipated, and as illustrated by the studies here, the immunological outcome following its inactivation appears to be dependent on the nature of the antigenic challenge.

A provocative question that comes to mind is whether abnormal T cell AICD in *Ccr2*-null mice constitutes an important pathogenic feature of the CIA phenotype in these mice. We have insufficient evidence to conclusively link decreased AICD and enhanced susceptibility to CIA, including T cell and B cell activation and increased anti-CII Ab production in this model. Nevertheless, it is notable that inhibition of T cell apoptosis has been associated previously with both RA and CIA (66, 67). In addition, RA patients have a subset of $CD4^+CD28^-$ T cells in the peripheral blood that frequently undergoes clonal expansion and is resistant to apoptosis, and additionally, Bcl-2 and related antiapoptotic proteins are upregulated in the T cells of RA patients (66). In this light, we speculate that impaired T cell AICD observed in $Ccr2^{-/-}$ mice may contribute to an increased autoreactivity by enhancing T cell help to B cells for Ab production in CIA.

It is noteworthy that our in vivo proof-of-principle studies in *Ccr5*-null mice do not support the prevailing notion that CCR5 is a central player in RA pathogenesis (3, 68–71). This finding should be interpreted in light of the observation that CCR5 is not the only chemokine receptor whose expression is elevated in RA. For example, invariably both CCR5 and CXCR3 are elevated in RA (68, 69, 72). Furthermore, although in the CIA model, chemical antagonism of CCR5 leads to partial protection against the disease, it is important to note that the antagonist used in these studies blocks not only CCR5 but also CXCR3 (7), making it difficult to ascribe the protective effects solely to the antagonism of CCR5. Supporting our findings that CCR5 may play a redundant role in RA pathogenesis are the observations of Santiago et al., who showed that the leukocyte chemoattractant activity in RA synovial fluid was not dependent on the expression of CCR5 (73).

In summary, our findings emphasize the importance of conducting proof-of-concept studies using chemokine receptor knockouts to delineate the full repertoire of their redundant and nonredundant functions in diverse disease states, and emphasizes that the nature of the Th phenotype, at least in *Ccr2*-null mice, may be highly dependent on the nature of the antigenic challenge. Our findings of increased monocyte recruitment, enhanced susceptibility to arthritis, and autoantibody production following genetic inactivation of *Ccr2* suggest that additional research is required before therapeutic manipulation of distinct members of the chemokine system can be safely accomplished.

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