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Overexpression of CXC Chemokine Ligand 14 Exacerbates Collagen-Induced Arthritis

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CXCL14 is a relatively new chemokine with unidentified receptor and undefined function. Recently, we found that CXCL14 is upregulated in arthritic joints in a mouse model of autoimmune arthritis, collagen-induced arthritis. To examine the role of CXCL14 in the development and pathogenesis of autoimmune arthritis, we have generated transgenic (Tg) mice that overexpress CXCL14 under control of phosphoglycerate kinase promoter. The results showed that CXCL14-Tg mice developed more severe arthritis compared with wild-type controls. The draining lymph nodes of CXCL14-Tg mice were significantly enlarged and contained an increased number of activated T cells, particularly the CD44+CD62Llow effector memory cells. In addition, T cells from CXCL14-Tg mice exhibited an enhanced proliferative response against collagen II and produced higher levels of IFN-γ. These results indicate for the first time that CXCL14 plays a role in the autoimmune arthritis, which may have an implication in understanding the pathogenic mechanisms of rheumatoid arthritis in humans and, ultimately, therapeutic interference. The Journal of Immunology, 2010, 184: 000–000.

Materials and Methods

Generation of CXCL14 Tg mouse lines

Mouse CXCL14 cDNA containing 3’ flag tag (aggcgaggtctagaggaaag) was amplified by PCR using specific primers (5’-CAGAATTCCTCAGAT-AGGGCTCTCTGCGGCGCCG-3’) and (5’-GGGGAATTCCTACACGCTGCGTGTATCTTATCGTGCATCTTTGTAATGACGACCGGCTGGCTTCGCTTCTGG-3’) with C57BL/6 cDNA as template. The fragment was digested with EcoRI and inserted into the kpnA vector downstream of PGK promoter. The DNA fragment was gel purified, linearized, and microinjected into the pronucleus of fertilized eggs of C57BL/6 mice (Transgenic Mouse Facility at Baylor College of Medicine, Houston, TX). The following two sets of primers were used to detect CXCL14 transgene in tail DNA: 5’-CAGAATTCCTCAGATTAGGCTCTCTGCGGCGCCG-3’ and 5’-GGGGAATTCCTACACGCTGCGTGTATCTTATCGTGCATCTTTGTAATGACGACCGGCTGGCTTCGCTTCTGG-3’.

CXCL14 expression by ELISA and real-time PCR

Five mice from each group were used to measure CXCL14 expression by ELISA. Briefly, the tissue lysates were prepared by homogenization in lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.2% sodium deoxycholate, 1 mM sodium ethylene diamine tetraacetate, and 1 mM phenylmethylsulfonyl fluoride). Tissue and cell debris were removed by centrifugation at 10,000 rpm for 5 min. Protein concentration was determined with a spectrophotometer. ELISA plates were coated with 10 µg/ml monoclonal anti-FLAG Ab (Sigma-Aldrich, St. Louis, MO). Tissue lysates from different organs of wild-type and CXCL14-Tg mice were diluted to 0.3 mg/ml of protein concentration and added into the plates. Biotin-conjugated polyclonal anti-mouse CXCL14 (R&D Systems, Minneapolis, MN) was used as the detecting Ab. The Journal of Immunology, 2010, 184: 000–000.

Abbreviations used in this paper: CIA, collagen-induced arthritis; CII, collagen II; DC, dendritic cell; PGK, phosphoglycerate kinase; Tg, transgenic.
**Induction of CIA and evaluation of arthritis**

Wild-type and CXCL14-Tg mice on C57BL/6 background (8–12 wk old, both sexes) were immunized as previously described (10, 11). Briefly, mice were injected intradermally at the base of the tail with 100 μg (in 100 μl) chicken collagen II (CII) (Sigma-Aldrich) dissolved in 0.01 M acetic acid and emulsified in an equal volume of CFA prepared by grinding 100 mg heat-killed *Mycobacterium tuberculosis* (H37Rv; Difco Laboratories, Detroit, MI) in 20 ml IFA (Sigma-Aldrich). Three weeks after primary immunization, mice were given the same injection. Mice were observed for the onset of arthritis, and an arthritis index was derived by grading the severity of each paw as described (12). Each paw was scored based on the degree of swelling and periarticular erythema using a scale of 0–3 as follows: 0 = no evidence of erythema or swelling; 1 = erythema confined to one joint region only; 2 = erythema and swelling limited to one joint region only; and 3 = severe erythema and swelling extending from the ankle to the midfoot (tarsal) joint, involving both joint regions. Scores from all four paws were added to provide the total score for each mouse. The maximum possible score per mouse was 12. The incidence was expressed as the percentage of mice that showed visible symptoms of arthritis.

**Flow cytometry**

Single-cell suspensions of draining lymph nodes from wild-type and CXCL14-Tg mice with CIA were stained with different Abs to cell surface markers. Abs to CD3, CD4, CD8, CD44, CD62L, B220, and CD19 were all purchased from BD Pharmingen (San Diego, CA). The samples were acquired by an FACSCalibur flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Detection of anti-CII Abs by ELISA**

CII-specific Abs in mouse sera were determined by ELISA as described (11). Briefly, microplates were coated with chicken CII overnight and then blocked with 10% FCS. Serum samples were added and incubated for 1 h at 37˚C and followed by addition of HRP-conjugated goat anti-mouse IgM, IgG1, and IgG2a (Southern Biotech, Birmingham, AL).

**Cytokine assays**

Draining lymph node cells pooled from the same group of mice were either unstimulated or stimulated with different concentrations of chicken CII and cultured in 24-well plates for 3 d. The supernatants were used to determine the levels of various cytokines using ELISA kits (BD Pharmingen) according to the manufacturer’s instructions.

**Cell proliferation**

Cells of draining lymph nodes were cultured in vitro with various concentrations of CII for 3 d and pulsed with [3H]thymidine (1 μCi/well) for the last 18 h. Cellular proliferation was determined by [3H] incorporation.

**Statistical analysis**

Student t test (GraphPad Prism, GraphPad, San Diego, CA) was used to determine the significance of differences in means. p values less than 0.05 were considered to be statistically significant.

**Results**

**Generation of CXCL14-Tg mice**

CXCL14 is expressed in different tissues including the joints, but the expression pattern of CXCL14 in different cell lineages is not clear. To understand the role of CXCL14 in inflammatory responses, we wanted to overexpress CXCL14 in all cell lineages using a ubiquitous PGK promoter. Mouse CXCL14 cDNA containing 3’ flag tag was inserted into the kbpA vector downstream of PGK promoter. To determine the expression of the transgene, tissue lysates from different organs of wild-type and CXCL14-Tg mice were assayed by ELISA with anti-FLAG Ab-coated plates. The results showed that there was significant expression of CXCL14-FLAG in all of the tissues from CXCL14-Tg mice, but the fusion protein was not detected in the tissues of wild-type mice (Fig. 1A). To further examine the overexpression of CXCL14 in the Tg mice, total RNA was extracted from different tissues of CXCL14-Tg mice and wild-type controls and subjected to real-time PCR assay. The levels of CXCL14 were increased by approximately 3- to 6-fold in different tissues tested in CXCL14-Tg mice compared with wild-type mice (Fig. 1B), confirming an overexpression of CXCL14 in CXCL14-Tg mice.
We analyzed lymphoid tissues for cellular composition and found no alterations in the numbers of lymphocytes, DCs, and macrophages in bone marrow, thymus, spleen, and lymph nodes in unmanipulated CXCL14-Tg mice (data not shown).

**Exacerbated CIA in CXCL14-Tg mice**

Because we have previously found that CXCL14 is significantly upregulated in the arthritic joints of mice with CIA (9), we wanted to investigate whether overexpression of CXCL14 affects the development and pathological outcome of CIA. CXCL14-Tg mice and wild-type controls were used to induce CIA. The results showed that CXCL14 overexpression led to an accelerated onset of the disease and increased disease incidence (Fig. 2). By day 17 after immunization, all CXCL14-Tg mice developed CIA, whereas only 75% of control mice had the disease (Fig. 2B). In addition, CXCL14-Tg mice developed more severe CIA than wild-type control mice (Fig. 2A). Thus, our results indicate that CXCL14 overexpression promotes the development of CIA.

**Increased severity of CIA in CXCL14-Tg mice is associated with an increased T cell response**

We assessed the effect of CXCL14 overexpression on T cell activation and proliferation. Interestingly, the draining (inguinal) lymph nodes recovered from CXCL14-Tg mice were significantly increased in their sizes (Fig. 3A) and cell numbers (Fig. 3B) compared with those in the control mice. Because the percentages of CD3+, CD4+, and CD8+ T cells were similar between wild-type and CXCL14-Tg mice (data not shown), both CD4+ and CD8+ T cells were proportionally increased in CXCL14-Tg mice. In addition, flow cytometry analysis shows that the percentages of effector memory CD4+ T cells (CD44+CD62Llow) out of total CD4+ T cells were significantly increased in CXCL14-Tg mice (Fig. 3C, 3D). Because total lymph node cell numbers were increased in the Tg mice, the increase in the percentages of effector memory T cells indicate a significant expansion of this population in CXCL14-Tg mice. Although the percentages of central memory CD4+ T cells (CD44+CD62Lhigh) within CD4+ T cells were similar between

**FIGURE 3.** Overexpression of CXCL14 enhances T cell priming and expansion. CXCL14-Tg and wild-type control mice with CIA were sacrificed for analysis. A, Sizes of draining lymph nodes. B, Total cell numbers of the draining lymph nodes. C, Lymph node cells were analyzed by flow cytometry. CD3+CD4+ T cells were gated and further analyzed for CD44 and CD62L expression. D, The percentages of CD44high CD62Llow cells of total CD4+ T cells from wild-type mice and CXCL14-Tg mice were compared. E, Draining lymph node cells were restimulated with CII and harvested 72 h later in the presence of [3H]thymidine for the last 18 h. IFN-γ levels in the supernatant after 3 d culture with CII. Values are the mean ± SEM with triplicates. Data are representative of two independent experiments with similar results. Open circles indicate wild-type mice; solid circles indicate CXCL14-Tg mice.

**FIGURE 4.** CXCL14 overexpression enhances autoantibody production in CIA mice. Serum samples were collected at peak time of CIA and measured for the levels of IgM (A), IgG1 (B), and IgG2a (C) isotypes of anti-CII autoantibodies by ELISA. Each dot represents one mouse. Student t test was used.
CXCL14-Tg mice and the controls (Fig. 3C), the cell number of this population was also proportionally increased in CXCL14-Tg mice.

We further examine Ag-specific cellular proliferation of T cells by in vitro restimulation with CII. Draining lymph node cells from CXCL14-Tg mice exhibited a significantly higher proliferative response compared with that of control mice (Fig. 3E). These results demonstrated that enhanced T cell activation and proliferation is present in CXCL14-Tg mice, which may drive more severe arthritogenic response in these mice.

CXCL14-Tg mice exhibit enhanced Th1 cytokine production
CIA has long been considered a Th1-mediated inflammatory disease (13–15), and Th17 cells have recently been indicated to be involved in the pathogenesis of autoimmune arthritis (16, 17). To examine whether CXCL14 overexpression affects cytokine profile, we measured cytokine production by lymph node cells from CXCL14-Tg mice and controls. Our results show that lymph node cells from CXCL14-Tg animals produced significantly higher levels of IFN-γ upon in vitro restimulation with the immunizing Ag CII (Fig. 3F). A decrease in IFN-γ production in the cultures with higher dosages of CII was observed, which may be due to the acidic effect of CII. There were no significant differences in the levels of IL-4, IL-10, and IL-17 between cell cultures from CXCL14 mice and wild-type controls (data not shown).

CXCL14 overexpression enhances autoantibody production
Because anti-CII Abs play an important role in the pathogenesis of CIA (18, 19), we determined whether CXCL14 overexpression affected the levels of CII-specific Abs. The serum levels of IgM, IgG1, and IgG2a CII-specific Abs were evaluated by ELISA. Our results showed that CXCL14-Tg mice produced higher levels of CII-specific Abs than control mice (Fig. 4). Particularly, IgM and IgG2a isotypes of anti-CII Abs were significantly elevated in CXCL14-Tg mice. The increase of IgG2a Ab levels is consistent with the elevated Th1 cytokine production in CXCL14-Tg animals, indicating a predominant Th1-mediated response (20). The increased levels of IgM and IgG2a autoantibodies may contribute to the increased severity of CIA in CXCL14-Tg mice.

CXCL14 expression in arthritic joints of CXCL14-Tg mice
To determine whether CXCL14 is significantly elevated in the arthritic joints of the Tg mice, joint tissues were isolated and subjected to real-time PCR. Compared to the expression levels of CXCL14 in the joints from naive mice, the expression of CXCL14 in arthritic joints in both wild-type mice and CXCL14-Tg mice were increased by more than 20-fold (Fig. 5). Although the expression levels of CXCL14 in the arthritic joints of CXCL14-Tg mice were increased compared with those in the arthritic joints of wild-type mice, the difference was not statistically significant (Fig. 5).

Discussion
Until now, the function of CXCL14 was largely unknown. In the current study, our results demonstrated that overexpression of CXCL14 promoted T cell activation and Th1 differentiation and led to an exacerbated arthritogenic response. However, the mechanisms underlying the enhanced T cell response and exacerbated arthritis in CXCL14-Tg mice remain to be investigated. We propose several possibilities. First, overexpression of CXCL14 may promote DC maturation and activation, which in turn promotes T cell activation. Previous results show that CXCL14 is a chemoattractant and activator of DCs and may be involved in DC homing in vivo (6, 8). Although a recent report showed that, using CXCL14-deficient mice, CXCL14 is dispensable for DC function and localization within peripheral tissues (21), it may reflect a functional redundancy. Second, it has been shown that CXCL14 may be involved in the generation of tissue macrophages (22). Overexpression of CXCL14 may greatly promote this process, ultimately resulting in more inflammatory cytokine secretion. Finally, because CXCL14 is a chemokine, its overexpression is likely to affect cell migration, particularly the migration of DCs and macrophages, as previous studies indicated (6–8, 22).

Our data indicated that CXCL14 expression in the joint of unimmunized CXCL14-Tg mice was significantly increased compared with that in the joint of unimmunized wild-type mice. This increase may play a role in cell infiltration into the joints at the early initiation phase of CIA. At the peak time of CIA, the expression levels of CXCL14 in the joints were greatly increased compared with the CXCL14 expression levels in the normal joints. But the differences between the expression levels of CXCL14 in arthritic joints from control animals and CXCL14-Tg mice were not significant. The results suggested that the increased levels of CXCL14 expression in the Tg mice may have more profound effects at the beginning of the inflammatory response, leading to the elevated arthritogenic response in the Tg mice.

Although an accurate defining of the pathological role of CXCL14 requires thorough understanding of the underlying mechanisms, our findings indicated an important role of CXCL14 in the development of autoimmune arthritis. This finding may have an implication in understanding the pathogenesis of human rheumatoid arthritis as well as possible therapeutic interference.

Disclosures
The authors have no financial conflicts of interest.

References


