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CXCL13 Neutralization Reduces the Severity of Collagen-Induced Arthritis

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Objective. **To investigate the role of CXCL13 in the development and pathogenesis of collagen-induced arthritis (CIA), and to determine the mechanisms involved in the modulation of arthritogenic response by CXCL13 neutralization.**

Methods. **Mice were immunized with type II collagen (CII) and treated with anti-CXCL13 or control antibodies during boosting. Mice were monitored for the development and severity of arthritis. The effects of CXCL13 neutralization on immune response to CII were evaluated by cytokine production by CII-specific T cells and CII-specific antibody production. Follicular response in the spleen and in synovial tissue was determined by in situ immunohistology.**

Results. **Mice receiving neutralizing antibodies to CXCL13 developed significantly less severe arthritis compared with mice injected with phosphate buffered saline or control antibodies. Follicular response both in the spleen and in synovial tissue was inhibited by anti-CXCL13 treatment. Injection with anti-CXCL13 antibodies did not significantly affect antigen-specific recall lymphocyte proliferation or type 1 cytokine production in vitro. Antibody response specific to CII was not inhibited by anti-CXCL13 treatment. However, anti-CXCL13 treatment induced significantly higher levels of interleukin-10 production after in vitro CII stimulation.**

Conclusion. **Neutralization of CXCL13 inhibits the development of CIA and reduces follicular response in both lymphoid and nonlymphoid tissues. These findings may have important implications regarding the pathogenesis and treatment of autoimmune arthritis.**

The chemokine CXCL13 is constitutively produced by stromal cells, such as follicular dendritic cells, in lymphoid tissues and attracts naive B cells and certain activated and memory T cells in vitro (1–4). CXCL13 interacts with the receptor CXCR5, which is primarily expressed on mature B lymphocytes and Burkitt's lymphoma cells (5). Mice genetically deficient in either CXCL13 or CXCR5 lack most inguinal lymph nodes (LNs), possess few or abnormal Peyer's patches, and have an unorganized spleen (6,7).

When there is chronic inflammation, lymphocytes are not restricted to lymphoid tissues but often infiltrate and accumulate in affected tissues (8). These lymphocytic infiltrates, which are present in various affected nonlymphoid tissues, can form tertiary lymphoid structures by a process called lymphoid neogenesis or ectopic lymphoid organogenesis (9,10). These structures are found in many autoimmune diseases, including rheumatoid arthritis (RA) (9–14), Hashimoto thyroiditis (15,16), Sjögren's syndrome (17,18), and myasthenia gravis (19,20). In addition, in chronic infections such as *Helicobacter pylori* gastritis (21,22), hepatitis C (23,24), and *Borrelia burgdorferi*–caused Lyme disease (25), ectopic lymphoid structures can also be frequently found in affected tissues. It has been suggested that the process of lymphoid neogenesis from unorganized infiltrates to germinal center (GC) reaction in autoimmune diseases is associated with increased disease severity and accelerated breakdown in self tolerance (26).

The first experiment that demonstrated the connection between inflammation and lymphoid neogenesis was carried out by introducing a lymphotoxin α (LTA) transgene under the control of the insulin promoter, which induced organized ectopic GCs (27). In a similar

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system, transgenic expression of CXCL13 also induced the formation of compartmentalized ectopic lymphoid structures (28). The observation that LTA can induce the expression of an array of adhesion molecules and chemokines indicates the central role of LTA in lymphoid neogenesis (29). In addition, transgenic expression of LTA can induce ectopic expression of CXCL13 and CCL21 in mice (30). These findings demonstrate that the same molecular mediators that control lymphoid organogenesis are also involved in inflammation-associated lymphoid neogenesis.

CXCL13 is expressed in the synovial tissue of RA patients, particularly in the germinal centers, where it localizes with follicular dendritic cells (31). In mice with collagen-induced arthritis (CIA), we observed that CXCL13 expression was elevated in arthritic joints compared with that in normal joints (data not shown). It is still unclear how and why mononuclear infiltrates resembling secondary lymphoid organs develop in the synovial tissue of patients with RA and to what extent follicular response in the affected tissue participates in the inflammation process. To directly determine the role of CXCL13 activity and GC response in the pathogenesis of autoimmune arthritis, we used a CIA model (32) to examine the effect of CXCL13 neutralization on disease severity and its modulation of immune response to CII.

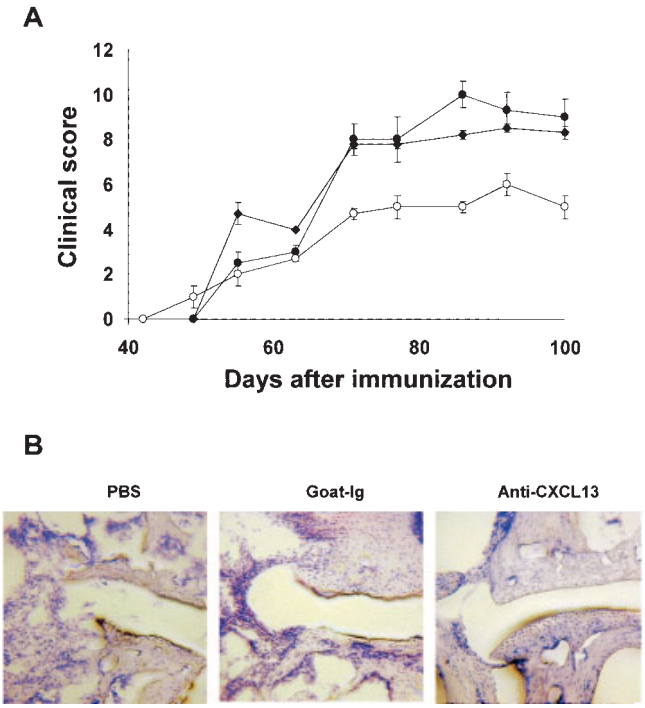

Figure 1. Amelioration of collagen-induced arthritis by CXCL13 neutralization. **A,** Male DBA/1 mice were immunized and boosted with bovine type II collagen (CII). Clinical scores and incidence of arthritis were recorded. The paws of mice treated with anti-CXCL13 antibodies (open circles), control Ig (solid circles), and phosphate buffered saline (PBS) (diamonds) were evaluated for the severity of inflammation, and combined clinical scores were recorded (mean ± SEM; n = 8). Data are representative of 2 independent experiments. **B,** Histologic examination of the joints of CII-immunized mice. Joints of anti-CXCL13–treated or control mice were processed and stained with hematoxylin and eosin (original magnification × 100).

MATERIALS AND METHODS

Mice. Male DBA/1 mice, 8–12 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME), housed in autoclaved microisolators, provided with sterile bedding, food, and water, and maintained on a 12-hour day/night cycle. Animal experimentation was performed in accordance with protocols approved by the Animal Research Committee of Baylor College of Medicine.

Induction of CIA and evaluation of arthritis. Mice (8 per group) were immunized with bovine CII to induce CIA, as previously described (32). The scoring system was based on the degree of swelling and periarticular erythema. Immunized animals developed CIA of various severities 4–5 weeks after the first immunization. The scores of all 4 paws were added to yield the arthritis index (33). In CXCL13 neutralization experiments, 1 day before boosting (day −1) and on days 2 and 5 after boosting, groups of mice were given 200 μg (in 200 μl phosphate buffered saline [PBS]) of anti-CXCL13 antibodies (R&D Systems, Minneapolis, MN), goat IgG, or an equal volume of PBS.

Immunohistology. The procedures for freezing tissues, sectioning, and immunohistochemical staining were conducted as previously described (32,34,35). Briefly, joint tissue samples, spleens, and draining LNs were frozen in OCT compound. Serial 6-μm-thick frozen sections were cut in a cryostat microtome, thaw-mounted onto poly-L-lysine–coated slides, air-dried, fixed in ice-cold acetone for 10 minutes, and stored at −80°C. Two GC B cell markers, peanut agglutinin conjugated with horseradish peroxidase (HRP) (Sigma, St. Louis, MO) and biotinylated GL-7 monoclonal antibody, were used to visualize GC formation in situ. Streptavidin–alkaline phosphatase (AP) conjugate (Southern Biotechnology, Birmingham, AL) was added following incubation with biotinylated antibodies. Bound HRP and AP activities were visualized with aminoethylcarbazole and naphthol-AS-MX phosphate/fast blue base (Sigma), respectively.

Detection of anti-CII antibodies. CII-specific antibodies in mouse sera were determined by enzyme-linked immunosorbent assay (ELISA), as previously described (33). Briefly, microplates were coated with bovine CII overnight and then blocked with 10% fetal calf serum. Samples were added and incubated for 1 hour at 37°C and washed. HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgM (Southern Biotechnology) were used as secondary detection reagents. Antibody titers were determined as the end point dilutions when optical density values were ≥2.5-fold those of normal control sera on each plate.

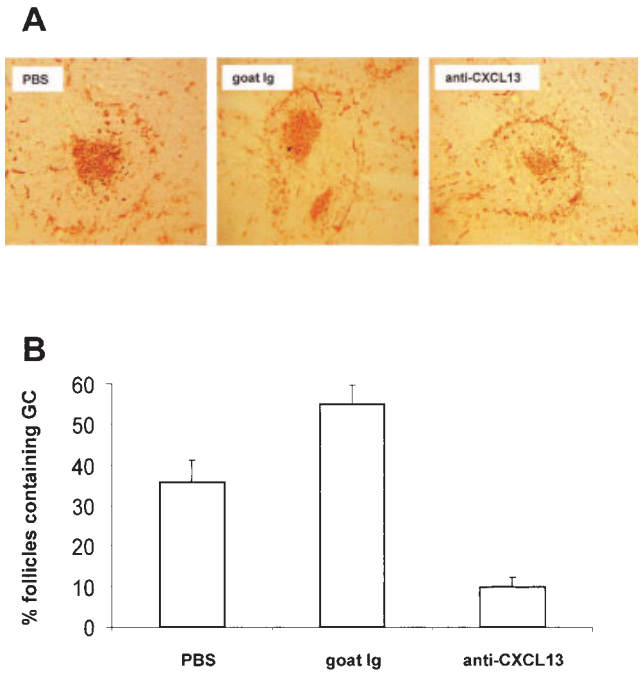

Figure 2. Inhibition of germinal center (GC) formation by CXCL13 neutralization. **A,** Sections of spleens obtained 6 weeks after primary immunization (3 weeks after boosting) were stained with the GC marker, peanut agglutinin (PNA) conjugated with horseradish peroxidase. GC areas are stained red (original magnification × 100). **B,** Splenic sections were stained for follicles with anti-B220 antibody and for GCs by PNA labeling. The numbers of individual follicles and GCs were counted. Values are the mean and SEM. PBS = phosphate buffered saline.

Cytokine assays. Murine interferon-γ (IFNγ), tumor necrosis factor α (TNFα), interleukin-4 (IL-4), and IL-10 were measured by ELISA using paired antibodies, according to the manufacturer's instructions (PharMingen, San Diego, CA).

RESULTS

Reduction of CIA severity by CXCL13 neutralization. To test our hypothesis that CXCL13 plays an important role in the pathogenesis of CIA and that neutralization of CXCL13 leads to an amelioration of the disease, we injected mice with neutralizing anti-CXCL13 antibodies and monitored the development of the disease over a period of 100 days. Our results showed that all mice in both the anti-CXCL13–treated group and the control antibody–treated group developed arthritis (data not shown), which reached a plateau at approximately the same time in both groups. However, the severity of arthritis that developed in mice that received anti-CXCL13 antibodies was significantly decreased compared with that in mice that received PBS or control goat Ig (Figure 1). The mean ± SEM peak clinical scores of arthritis were 10 ± 0.6 and 6 ± 0.5 in mice treated with control Ig and anti-CXCL13, respectively (Figure 1A). Consistent with this, histologic studies showed that anti-CXCL13 treatment reduced cellular infiltration in arthritic joints and reduced the degree of cartilage/bone erosion (Figure 1B). Thus, our data demonstrated a therapeutic effect of CXCL13 neutralization in CIA.

Reduction of follicular response in lymphoid and synovial tissues by CXCL13 neutralization. To assess the in vivo effect of anti-CXCL13 treatment on GC reaction, we examined GC formation in spleen and joint tissues 6 weeks after primary immunization. We found that GC formation in the spleens of mice treated with anti-CXCL13 antibodies was significantly decreased compared with that in control mice (Figure 2). In control mice, ~55% of the splenic follicles contained GCs, whereas only 10% of the follicles in the spleens of anti-CXCL13–treated mice contained GCs (Figure 2B). Furthermore, the size of GCs formed in the spleens of

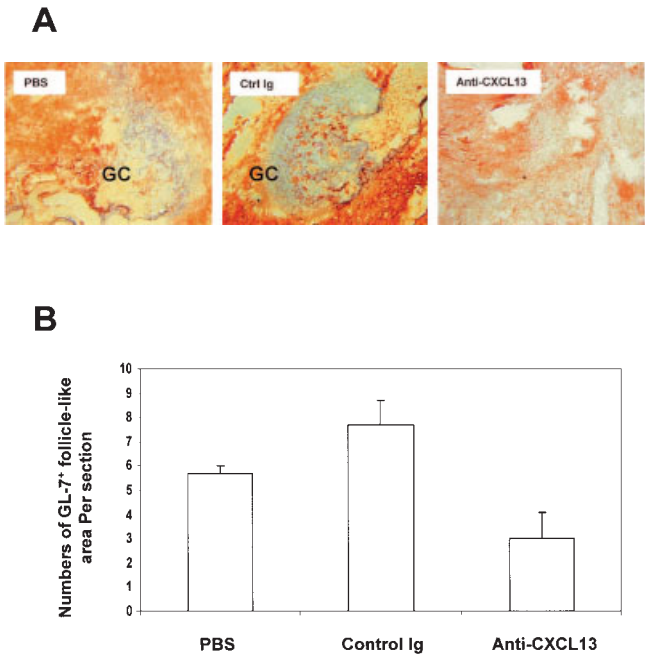

Figure 3. Inhibition of lymphoid neogenesis in joints by CXCL13 neutralization. **A,** Sections of joints from mice treated with phosphate buffered saline (PBS), control Ig, or anti-CXCL13 antibodies were stained to visualize germinal center (**GC**) B cells (red; original magnification × 200). **B,** Mean and SEM number of GL-7$^+$ follicle-like structures per joint section. Data are from 4 sections of affected paws of individual mice in each group.

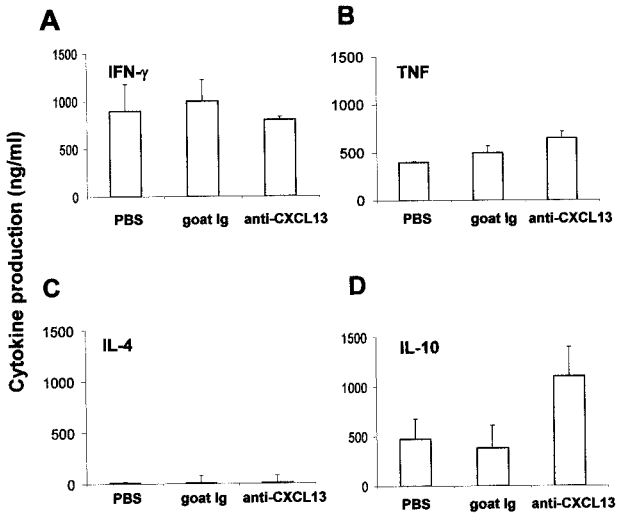

Figure 4. Cytokine production by lymph node cells in vitro. Six weeks after type II collagen (CII) immunization, draining lymph node cells from mice of different groups were cultured with 30 μg/ml of CII for 96 hours, and interferon-γ (IFNγ) (**A**), tumor necrosis factor α (TNFα) (**B**), interleukin-4 (IL-4) (**C**), and IL-10 (**D**) concentrations were measured by enzyme-linked immunosorbent assay. Values are the mean and SEM. Data are representative of 2 independent experiments with 6–8 mice in each group. PBS = phosphate buffered saline.

anti-CXCL13–treated mice was significantly smaller than those formed in control mice (Figure 2A).

We also found that neutralization of CXCL13 inhibited ectopic follicular formation in arthritic joints. Follicular structures were frequently observed in the inflamed joints of control mice (Figure 3). However, the formation of follicular structures in the joints of mice treated with anti-CXCL13 antibodies was significantly inhibited compared with that of mice treated with PBS or control Ig (Figure 3). Therefore, our data suggest that CXCL13 neutralization may inhibit CIA by suppression of follicular responses in lymphoid tissues and locally inflamed sites.

Effect of CXCL13 neutralization on antigen-specific cellular proliferation and type 1 cytokine production. To further determine the mechanisms responsible for the decreased arthritogenic response caused by anti-CXCL13 treatment, we examined the T cell response by determining antigen-specific T cell proliferation and cytokine production in vitro. Our results showed that antigen-specific proliferation of splenic cells or draining LN cells was similar between mice treated with anti-CXCL13 antibodies and those treated with control Ig, when restimulated with CII in vitro (data not shown). Production of IFNγ and TNFα, the 2 most important type 1 cytokines in autoimmune arthritis, was comparable between LN cells from anti-CXCL13–treated and control Ig–treated mice (Figures 4A and B).

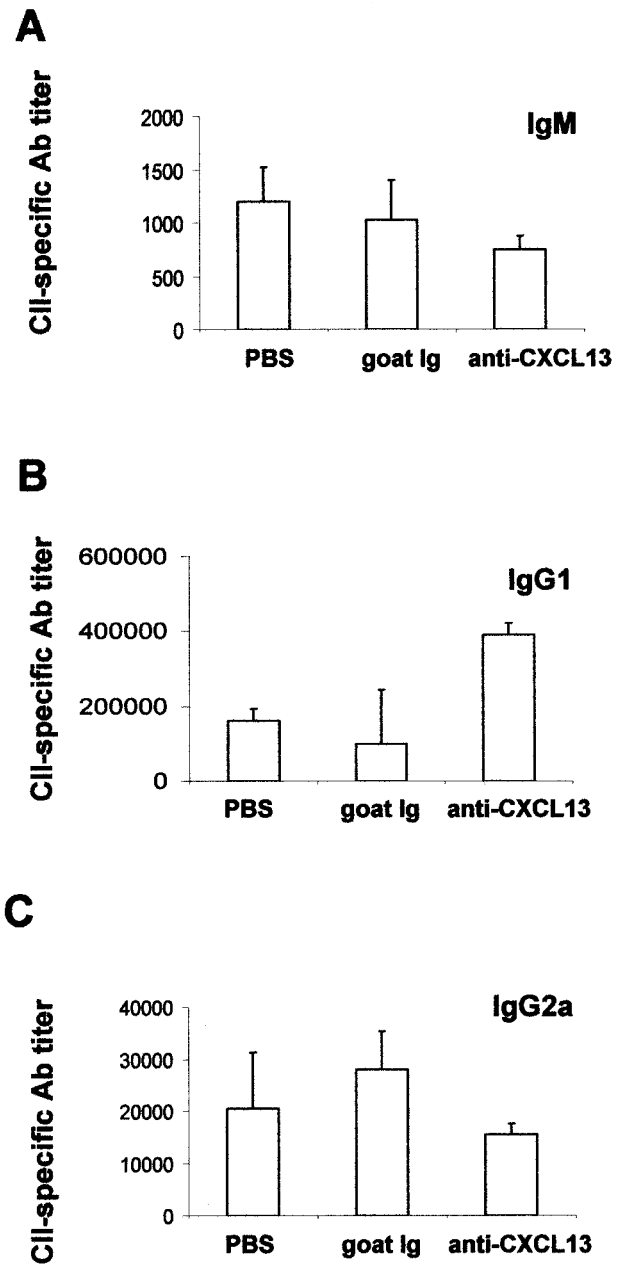

Figure 5. Effect of CXCL13 neutralization on specific antibody responses. Mice of various groups were bled 6 weeks after immunization, and serum antibodies (Ab) against type II collagen (CII) were measured by enzyme-linked immunosorbent assay. CII-specific IgM (**A**), IgG1 (**B**), and IgG2a (**C**) antibody titers are expressed as the mean and SEM. Data are representative of 2 independent experiments with 6–8 mice in each group. PBS = phosphate buffered saline.

The IL-4 level was very low in all groups (Figure 4C). However, there was a significant increase in IL-10 production by LN cells from mice treated with anti-CXCL13 antibodies compared with those from control mice (Figure 4D).

Production of CII-specific antibodies not inhibited by CXCL13 neutralization. Since it has been shown that anti-CII antibodies play an important role in the pathogenesis of CIA (36–39), we further investigated whether neutralization of CXCL13 affected the levels of CII-specific antibodies in animals immunized with CII. Serum levels of IgM, IgG1, and IgG2a CII–specific antibodies were evaluated by ELISA. Our results showed that anti-CXCL13 treatment did not inhibit the production of CII-specific antibodies of all Ig isotypes tested (Figure 5). In fact, there was an increase in the IgG1 CII–specific antibody levels in mice treated with anti-CXCL13 antibodies (Figure 5B). Thus, these data indicate that the inhibition of CIA by neutralization of CXCL13 was not caused by the suppression of specific antibody responses.

DISCUSSION

Although CXCL13 has long been suspected as an important mediator in certain autoimmune disorders, particularly RA, the results presented here are among the first to directly demonstrate its role in the development and pathogenesis of autoimmune arthritis in an animal model.

Our results demonstrate that neutralization of CXCL13 activity at the boosting stage significantly suppresses the disease severity over a long period of time. One possible mechanism for this suppression of CIA severity is the inhibition of follicular responses. Since CXCR5, the receptor for CXCL13, is expressed on B cells as well as on follicular T cells that provide helper function for antibody production (40–42), neutralization of CXCL13 impairs the homing and retention of both B cells and T helper cells to and in the follicles. Indeed, GC formation in mice receiving anti-CXCL13 antibodies was significantly inhibited compared with that in control mice. However, the overall production of CII-specific antibodies was not suppressed in mice treated with anti-CXCL13 antibodies (Figure 5). One possible explanation for this is the timing of antibody administration. Since anti-CXCL13 was administered 20 days after primary immunization, at a time when antigen-specific lymphocytes had already been properly primed and activated, neutralization of CXCL13 would not significantly affect the ongoing immune responses. Thus, systemic inhibition of immune responses is unlikely to be the major mechanism responsible for the amelioration of CIA induced by anti-CXCL13 treatment at the boosting stage.

Importantly, it has been shown that, in the ectopic lymphoid structures in the synovial tissues of RA patients, diversification of the B cell repertoire by Ig hypermutation and the differentiation of activated B cells into plasmacytes and memory cells can take place (43,44), indicating that this response is part of the chronic process of this disease. The process of lymphoid neogenesis in autoimmune diseases is associated with increased disease severity and accelerated breakdown in self tolerance (45). Thus, it would be interesting to determine whether treatment with anti-CXCL13 antibodies suppresses the formation of ectopic lymphoid follicles in CIA. Significantly, our results showed that neutralization of CXCL13 inhibited the formation of ectopic lymphoid follicles in the joints (Figure 3). These results suggest that suppression of lymphoid neogenesis may be an important mechanism in the in vivo effects of CXCL13 neutralization. Further study is needed to establish the direct association between lymphoid neogenesis and pathogenesis by manipulating lymphoid neogenesis with other reagents that are known to affect GC reaction.

One unexpected effect of in vivo anti-CXCL13 administration was the enhanced production of IL-10 by cell cultures from anti-CXCL13–treated mice. Since IL-10 is generally considered to be an antiinflammatory cytokine, elevated production of IL-10 may be another mechanism that contributes to suppression of CIA in anti-CXCL13–treated mice. Interestingly, in cell cultures from mice immunized with CII and treated with the steroid prednisolone, although IFNγ production was inhibited, the level of IL-10 was significantly enhanced (data not shown). Thus, the role of regulatory T cells in the attenuation of the arthritogenic response by CXCL13 neutralization should be investigated.

Overall, our results indicate that CXCL13 plays an important role in the development and pathogenesis of CIA, and targeting CXCL13 activity can convey a beneficial effect in autoimmune arthritis. Thus, the current study may have important implications for the treatment of RA as well as other autoimmune disorders.

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