

Interleukin-17 as a Molecular Target in Immune-Mediated Arthritis

Immunoregulatory Properties of Genetically Modified Murine Dendritic Cells That Secrete Interleukin-4

Sujata Sarkar, Laura A. Tesmer, Vindhya Hindnavis, Judith L. Endres, and David A. Fox

Objective. Our previous studies have shown that murine dendritic cells (DCs) genetically modified to express interleukin-4 (IL-4) reduce the incidence and severity of murine collagen-induced arthritis. The present studies were performed to assess the immunoregulatory mechanisms underlying this response, by assessing the effects of IL-4 DCs on cytokine production by subsets of T helper cells.

Methods. Male DBA mice ages 6–8 weeks old were immunized with type II collagen. Splenic T cells obtained during the initiation phase and the end stage of arthritis were cultured with IL-4 DCs or untransduced DCs in the presence of collagen rechallenge. Interferon- γ (IFN γ) and IL-17 responses were measured. Antibodies to IL-4, IL-12, and IL-23, and recombinant IL-4, IL-12, and IL-23 were used to further study the regulation of T cell cytokine production by IL-4 DCs.

Results. Splenic T cells obtained during the initiation phase of arthritis produced less IL-17 when cultured in the presence of IL-4 DCs, despite their production of increased quantities of other proinflammatory cytokines (IFN γ and tumor necrosis factor). T cell IL-17 production after collagen rechallenge was not inhibited by a lack of IL-23, since IL-4-mediated suppression of IL-17 was not reconstituted by IL-23, an otherwise potent inducer of IL-17 production by T cells. Although

IL-4 DCs can produce increased quantities of IL-12 and IFN γ , suppression of IL-17 production by IL-4 DCs was independent of both. While IL-17 production by T cells obtained during the initiation phase of arthritis was regulated by IL-4 DCs, IL-17 production by T cells obtained during end-stage arthritis was not altered.

Conclusion. Our data suggest that IL-4 DCs exert a therapeutic effect on collagen-induced arthritis by targeting IL-17. IL-17 suppression by IL-4 DCs is robust and is not reversed by IL-23. Timing might be important in IL-17-targeted therapy, since IL-17 production by T cells obtained during end-stage arthritis did not respond to suppression by IL-4 DCs.

Numerous cytokines are associated with the pathogenesis of rheumatoid arthritis (RA), including tumor necrosis factor (TNF), interferon- γ (IFN γ), interleukin-1 (IL-1), IL-6, IL-15, and IL-17. IL-17 is a proinflammatory cytokine that is secreted by T lymphocytes (1). Ligation of the IL-17 receptor, which is expressed on several cell types, including epithelial cells, endothelial cells, and fibroblasts, induces the secretion of IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein 1, prostaglandin E₂ (PGE₂), TNF α , and IL-1 β (2–5), as well as neutrophil chemotaxis and granulopoiesis (6,7). IL-17 induces the expression of matrix metalloproteinase 1 (MMP-1) and MMP-13 in RA synovial cells and osteoblasts (8,9). IL-17 is present in RA synovium (10) and induces the expression of RANKL, which contributes to bone resorption (11,12). Local overexpression of IL-17 increases the severity of murine arthritis, and neutralizing anti-IL-17 antibody reduces the severity of arthritis (13,14). IL-17-deficient mice have a reduced incidence and severity of collagen-induced arthritis (CIA) (15).

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Taken together, the available data suggest that IL-17 is important in both CIA and RA.

IL-23, a dimeric cytokine composed of the IL-12 p40 subunit and a unique p19 subunit (16), is secreted by dendritic cells (DCs) and induces the production of IL-17 by T cells (17,18). IL-23 p19^{-/-} mice are resistant to experimental autoimmune encephalomyelitis and CIA, suggesting that this cytokine is important in autoimmune diseases (19,20). There is some evidence that costimulatory molecules may also be involved in the regulation of IL-17, since IL-17 production is reduced in ICOS^{-/-} mice (21,22).

DCs are professional antigen-presenting cells that are capable of activating and regulating T lymphocytes (23). Previous studies in our laboratory have shown that a single injection of bone marrow-derived DCs genetically engineered to express IL-4 reduces the incidence and severity of CIA and alters primary immune responses in vivo (24,25). Such effects were not produced by T cells or by fibroblasts overexpressing IL-4, which suggests that the migratory properties of DCs are required. Although genetically modified DCs were only detectable in the spleen for a few days following injection, the effects of IL-4 DCs were long-lasting. Soluble IL-4 has a short half-life and has to be administered by continuous infusion or by repeated injections, whereas IL-4 DCs can be administered as a single injection and are effective in CIA, making this an attractive therapeutic approach. The molecular mechanisms by which IL-4 DCs alter an ongoing immune response in vivo are not yet understood in detail, although previous studies have shown that the polyclonal response of splenic T cells shifts to Th2 after administration of IL-4 DCs. However, there is no evidence for the suppression of IL-12 expression in IL-4 DCs despite a down-regulation of the expression of the IL-23 p19 subunit (25).

RA has been viewed as a Th1 disease, mediated in part by the expression and biologic effects of IFN γ . IFN γ , but not IL-4, is detected in the RA synovium (26). However, deficiency of either IFN γ or the IFN γ receptor causes an acceleration of CIA in mice (27). Furthermore, treatment of CIA with IL-4 DCs did not appear to deplete primed collagen II-specific Th1 cells in vivo (24). The existence of a third Th population, a distinct subset of IL-17-producing T cells (Th17), was recently proposed, which may be the pathogenic population in some autoimmune diseases (15,17,28,29). The present studies were performed to further our understanding of the mechanisms underlying the response to IL-4 DCs in CIA. We evaluated T lymphocyte responses to IL-4 DCs

during different phases of arthritis, and we found that IL-4 DCs selectively regulate the production of IL-17.

MATERIALS AND METHODS

Purification and stimulation of dendritic cells. Bone marrow-derived DCs and IL-4 DCs were derived from DBA mice as previously described (24). DCs were purified on day 5 of culture using CD11c Microbeads (Miltenyi Biotech). Dendritic cell purity was 90–99%. For some experiments, DCs and IL-4 DCs were then stimulated with 10 μ g/ml of soluble CD40L (Research Diagnostics, Flanders, NJ) for 24 hours, with or without 40 ng/ml of recombinant IL-4 (R&D Systems, Minneapolis, MN) added to the DCs or 10 μ g/ml of neutralizing antibody to IL-4 (clone 11B11; BD PharMingen) added to the IL-4 DCs. Culture supernatants were collected and frozen for later cytokine analysis.

Cytokine enzyme-linked immunosorbent assay (ELISA). Cytokine production in culture supernatants was analyzed by ELISA. Kits for measuring IL-6, TNF, IL-10, IFN γ , IL-4, and IL-17 were obtained from BD PharMingen (San Diego, CA).

Purification of T lymphocytes. Male DBA mice (The Jackson Laboratory, Bar Harbor, ME) ages 6–10 weeks were immunized at the base of the tail with an intradermal injection of chicken type II collagen and Freund's complete adjuvant (CFA; Chondrex, Redmond, WA). Mice were killed on day 14, and spleen and inguinal lymph nodes were harvested. Splenocytes were enriched for T lymphocytes using a pan-T cell isolation kit (Miltenyi Biotech, Sunnyvale, CA). T cell purity was 75–95%. Single-cell suspensions of lymph nodes were prepared and used in culture. CD4⁺ T cells were purified using EasySep kits from StemCell Technologies (Vancouver, British Columbia, Canada).

In vitro culture of T lymphocytes and DCs. T lymphocytes were cultured with DCs or IL-4 DCs at a ratio of 40:1 with anti-CD3 (clone 1452C11; BD PharMingen) or 100 μ g/ml of T cell culture-grade collagen (Chondrex) for 72 hours (anti-CD3) or 120 hours (collagen) in lymphocyte medium, as previously described (24). Culture supernatants were frozen, and cytokine levels were measured by ELISA. Neutralizing anti-IL-4 antibody (clone 11B11; BD PharMingen) was used at 1 μ g/ml, anti-p19 antibody (clone G23-8; eBioscience, San Diego, CA) at 5 and 10 μ g/ml, anti-IL-12 p70 antibody (clone 18.2; eBioscience) at 1 or 5 μ g/ml, and anti-IFN γ antibody (clone XMG1.2; BioLegend, San Diego, CA) at 1 or 10 μ g/ml. When IL-4 (R&D systems) was added to DCs, 10 ng/ml was used. IL-23 (eBioscience) was used at 10 and 100 ng/ml. IL-12 (eBioscience) was used at 1 ng/ml. Proliferation was assessed by the incorporation of ³H-thymidine.

Induction of arthritis in mice. Male DBA mice ages 7–9 weeks were housed under specific pathogen-free conditions. Chicken type II collagen was dissolved in 0.05M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C and was then emulsified in an equal volume of CFA. Collagen (100 μ g) was injected intradermally at the base of the tail. Arthritis was scored from day 21 using a scale of 0–4 per limb, where 0 = no swelling or redness, 1 = swelling or redness in 1 digit, 2 = mild swelling and redness involving the entire paw, 3 = moderate swelling and redness involving the entire

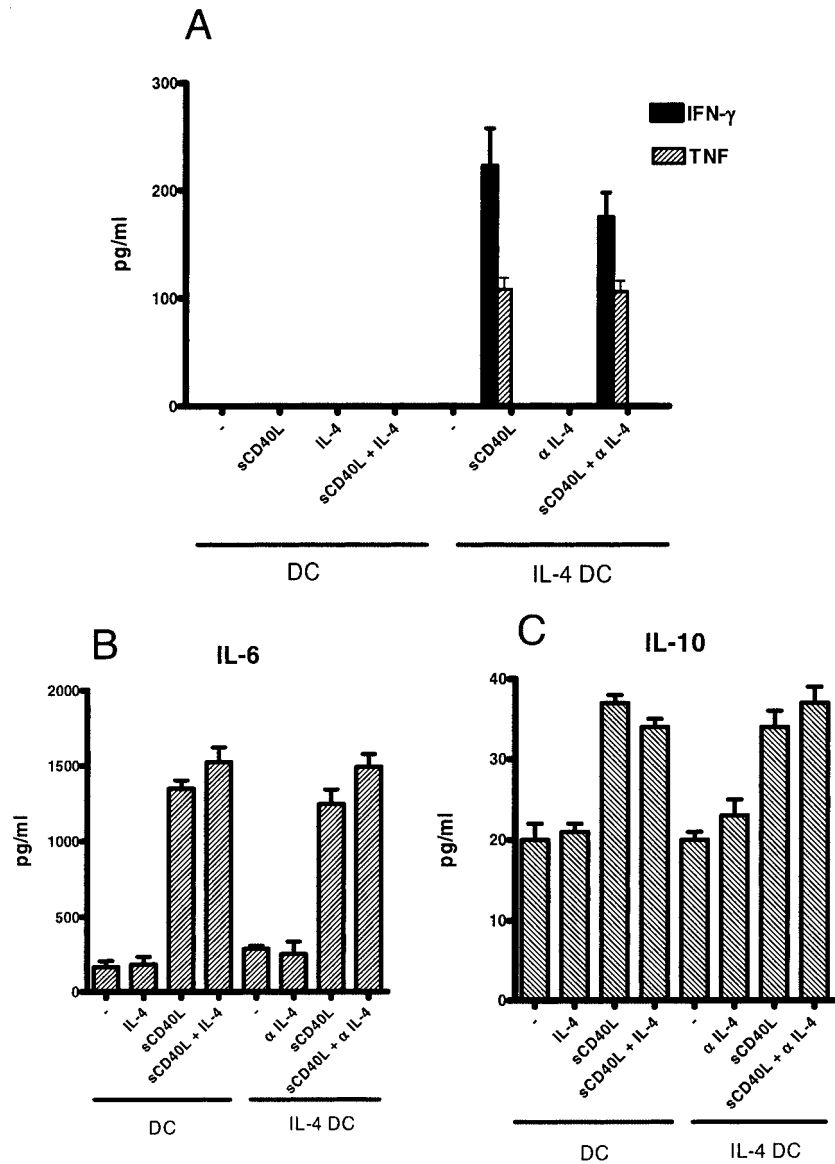


Figure 1. Cytokine production by interleukin-4 (IL-4) dendritic cells (DCs) and untransduced DCs after stimulation with CD40L. Untransduced DCs and IL-4 DCs were generated as described in Materials and Methods. DCs or IL-4 DCs (1×10^6 /ml) were stimulated with 10 μ g/ml of soluble CD40L (sCD40L) for 24 hours, with or without IL-4 (40 ng/ml) or neutralizing antibody to IL-4 (α IL-4; 10 μ g/ml), and supernatants were analyzed for **A**, tumor necrosis factor (TNF) and interferon- γ (IFN γ), **B**, IL-6, and **C**, IL-10 by enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 3 independent experiments.

paw, and 4 = severe swelling and redness involving the entire paw. Mice were considered arthritic if they had a score of 3 in at least 1 paw.

Mice with arthritis were killed on day 56, and T lymphocytes were isolated from spleens. T lymphocytes were then cultured with DCs or IL-4 DCs and collagen or anti-CD3.

Culture supernatants were collected at 72 or 96 hours and analyzed for cytokines by ELISA. All protocols involving mice were approved by the Committee on Use and Care of Animals, University of Michigan, Ann Arbor.

Statistical analysis. ELISAs were performed in triplicate. Data are presented as the mean \pm SEM. Statistical

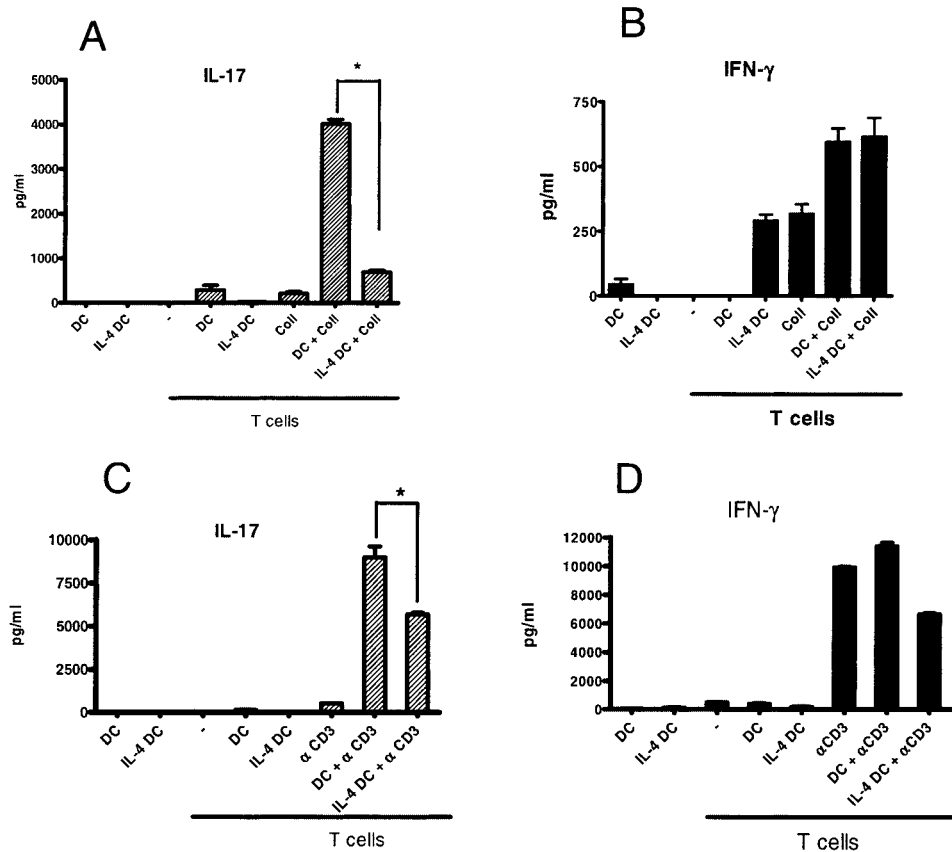


Figure 2. Role of interleukin-23 (IL-23) in IL-4 dendritic cell (DC)-mediated suppression of IL-17 production by T cells. T lymphocytes purified from the spleens of collagen-immunized mice were cultured with DCs or IL-4 DCs in the presence of **A** and **B**, collagen (Coll) or **C** and **D**, anti-CD3 (α CD3), and culture supernatants were collected after 5 days (for collagen) or 3 days (for anti-CD3) and analyzed by enzyme-linked immunosorbent assay for IL-17 and interferon- γ (IFN γ). Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 4 independent experiments. * = $P < 0.05$ by Student's t -test.

analyses were performed using Student's t -test or one-way ANOVA. P values less than 0.05 were considered statistically significant.

RESULTS

Cytokine profile of IL-4 DCs after stimulation with CD40 ligand. We evaluated the cytokine profile of IL-4 transduced DCs as compared with that of untransduced DCs. DCs and IL-4 DCs were generated as described in Materials and Methods. DCs or IL-4 DCs were stimulated for 24 hours with recombinant CD40L, with or without recombinant IL-4 (for DCs) or neutralizing antibodies to IL-4 (for IL-4 DCs). IL-4, in cultures of untransduced DCs, was used at 40 ng/ml, since IL-4 DCs produce as much as 40 ng/ml of IL-4 per 1×10^6 cells in 24 hours. Supernatants were analyzed for various cytokines by ELISA.

As shown in Figure 1A, the IL-4 DCs made more TNF and IFN γ than did the untransduced DCs after stimulation with CD40. These increases in TNF and IFN γ were independent of IL-4. DCs and IL-4 DCs produced similar quantities of IL-6 and IL-10 at baseline and after stimulation with CD40L, which was not altered by IL-4 (Figures 1B and C). Thus, immunoregulatory effects of IL-4 DCs occur despite their ability to produce augmented amounts of some proinflammatory cytokines.

IL-4 DC-induced decrease in IL-17 production by antigen-specific T cells. Experiments were next performed to evaluate the effects of IL-4 DCs on splenic T cells and draining lymph node cells during the induction phase of CIA. DBA mice were immunized with collagen and CFA intradermally at the base of the tail. Two weeks later, splenic T lymphocytes were isolated and

cultured with DCs or IL-4 DCs and anti-CD3 or collagen for 4–5 days. Culture supernatants were collected and analyzed by ELISA for IL-17 and IFN γ .

The results showed that T cells rechallenged with collagen in the presence of IL-4 DCs produced less IL-17 than did T cells cultured with untransduced DCs (Figure 2A). There was no change in IFN γ secretion from T cells when cultured with either untransduced DCs or IL-4 DCs (Figure 2B). Similar effects on IL-17 and IFN γ production were seen with lymphocytes from draining lymph nodes cultured with IL-4 DCs (data not shown). Such responses were not seen when an unrelated antigen, keyhole limpet hemocyanin, was used for challenge in collagen-immunized mice (data not shown). T cells or CD4 T cells cultured with DCs or IL-4 DCs at different ratios (20:1 or 40:1) yielded similar results (data not shown). Splenic T cells or CD4+ T cells cultured with IL-4 DCs as compared with DCs showed a slightly reduced proliferative response, but this difference was not statistically significant (data not shown). These data show that T cells produce IL-17 as an early response to collagen and that the production of IL-17 can be regulated by IL-4 DCs, even when IFN γ production by sensitized Th1 cells is refractory to regulation by IL-4 DCs.

We also analyzed the cytokine response of splenic T cells to a polyclonal stimulus, anti-CD3, in the presence of untransduced DCs or IL-4 DCs. T cells cultured with IL-4 DCs and anti-CD3 produced less IL-17 and less IFN γ than did T cells cultured with DCs (Figures 2C and D). Similar reductions in IL-17 and IFN γ production were observed with unfractionated splenocytes and with lymphocytes from draining lymph nodes (data not shown).

Effect of neutralizing IL-4 on the immunoregulatory effects of IL-4 DCs. T cells cultured in the presence of IL-4 DCs produced less IL-17, but IL-4 DCs have other alterations in gene expression beyond production of IL-4. We therefore studied the contribution of IL-4 to the immunoregulatory effects of IL-4 DCs by neutralizing IL-4 or by the addition of IL-4 to the cocultures with T cells. Splenic T cells from collagen-immunized mice were stimulated with collagen in the presence of untransduced DCs and recombinant IL-4 or in the presence of IL-4 DCs and neutralizing anti-IL-4 antibody. Culture supernatants were analyzed for IL-17. Addition of IL-4 to cultures with untransduced DCs reduced the level of IL-17 production by T cells. Neutralizing IL-4 in cultures containing IL-4 DCs resulted in increased production of IL-17 (Figure 3). This finding confirms that the IL-17 response in T cells is mediated by IL-4 that is secreted from IL-4 DCs.

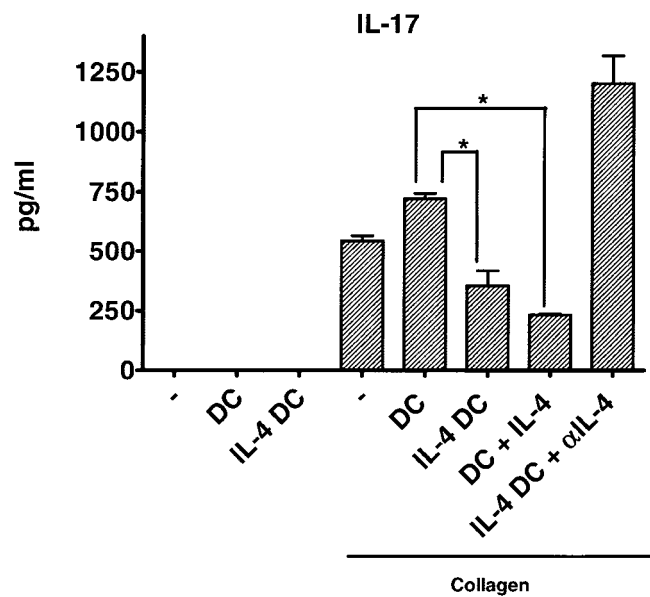


Figure 3. Effect of neutralizing or adding interleukin-4 (IL-4) on IL-17 production by T cells. Splenic T cells purified from collagen-immunized mice were cultured either alone (-), with dendritic cells (DCs), or with IL-4 DCs, with or without collagen. Anti-IL-4 antibody (α IL-4; 1 μ g/ml) or IL-4 (10 ng/ml) was added at the beginning of culture. Supernatants were collected after 4 days of culture and analyzed for IL-17 by enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 5 independent experiments. * = $P < 0.05$ by Student's *t*-test.

Role of IL-23 in IL-17 production by T cells in the presence of IL-4 DCs. While IL-23 is not necessary for the generation of IL-17 cells, it augments IL-17 production from memory Th17 cells (18). IL-23 is composed of 2 subunits, p19 and p40. Mice with the p19 knockout develop less severe arthritis (19). We next analyzed the role of IL-23 in IL-17 production in this system. We evaluated this by adding IL-23 or neutralizing antibody to the p19 subunit of IL-23 in cultures of T cells with IL-4 DCs and collagen. Our data showed that the addition of 100 ng/ml of IL-23 only modestly restored IL-17 production by T cells in the presence of IL-4 DCs (Figure 4A). The addition of 10 ng/ml of IL-23 had no effect on IL-17 levels (data not shown). Although IL-23 at 100 ng/ml resulted in some increase in the IL-17 response, the levels were still lower than those produced by T cells in the presence of untransduced DCs.

Neutralizing IL-4 in cultures containing IL-4 DCs resulted in a much greater increase in IL-17 as compared with reconstituting the IL-23 level in the presence of IL-4 DCs. The increased IL-17 production after neutralization of IL-4 was only partly reversed after neutralizing the IL-23 with antibody to the p19 subunit. As expected,

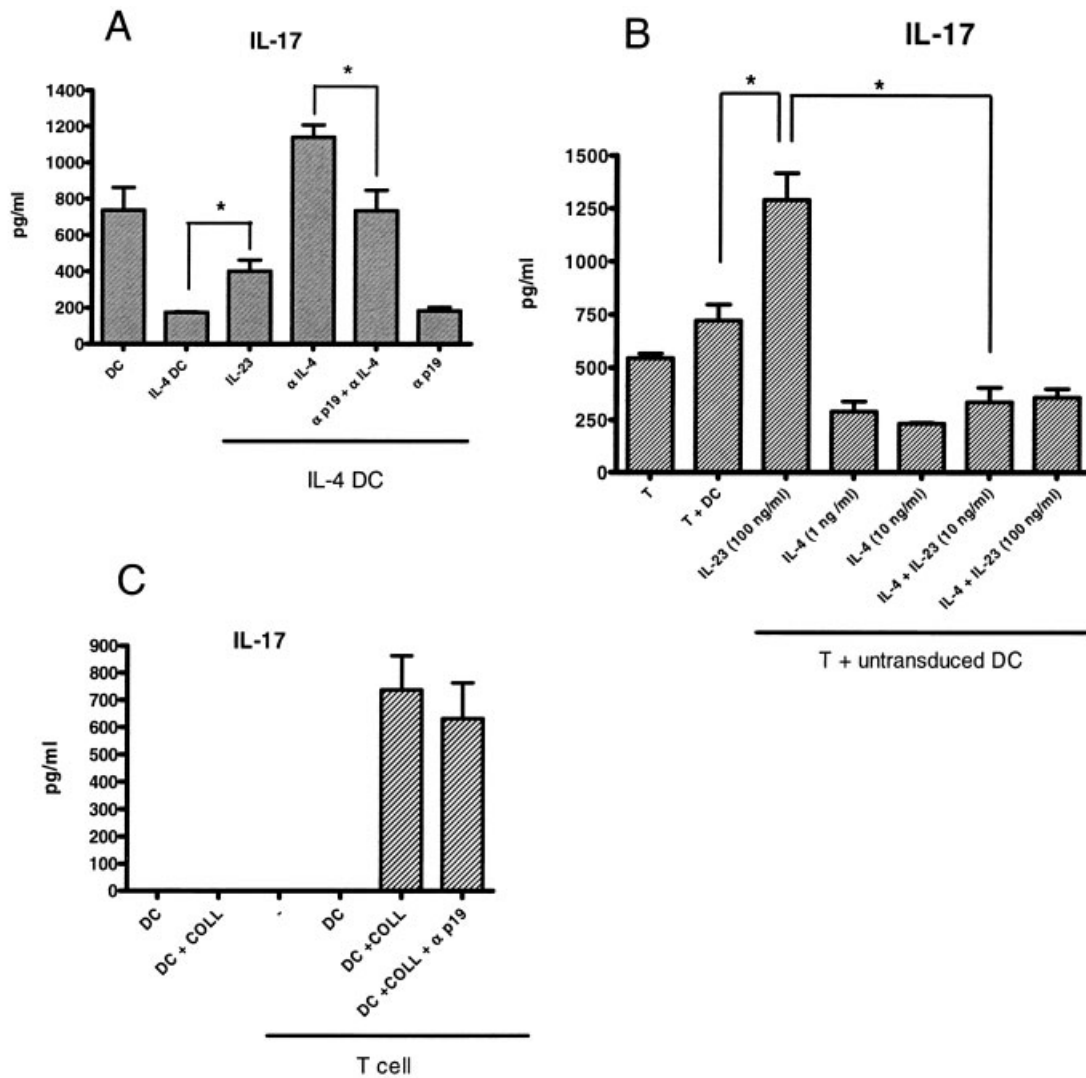


Figure 4. Suppressive effect of interleukin-4 (IL-4) dendritic cells (DCs) on IL-17 production by T cells is only partially mediated by underproduction of IL-23. **A**, Splenic T cells from collagen-immunized mice were cultured with collagen and either IL-4 DCs or untransduced DCs and then with neutralizing antibodies to IL-4 (α IL-4; 1 μ g/ml), IL-23 p19 subunit (α p19; 10 μ g/ml), or IL-23 (100 ng/ml). Supernatants were collected after 5 days of culture and analyzed for IL-17 by enzyme-linked immunosorbent assay (ELISA). **B**, Splenic T cells from collagen-immunized mice were cultured with DCs and collagen in the presence of IL-4 (1 or 10 ng/ml) and/or IL-23 (10 or 100 ng/ml). When both IL-23 and IL-4 were added, IL-4 was used at 10 ng/ml. Culture supernatants were collected after 5 days and analyzed for IL-17 by ELISA. **C**, Splenic T cells from collagen-immunized mice were cultured with untransduced DCs and collagen (Coll). Antibody to the p19 subunit of IL-23 (10 μ g/ml) was added at the initiation of culture. Supernatants were collected after 5 days of culture and analyzed for IL-17 by ELISA. Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 3 independent experiments. * = $P < 0.05$ by Student's *t*-test.

neutralizing antibody to the IL-23 p19 subunit did not alter IL-17 production by T cells in the presence of IL-4 DCs. Similar changes in IL-17 regulation were seen with IL-17 enzyme-linked immunospot (ELISpot) assays (data not shown).

These findings suggest that the suppression of IL-17 production by T cells in the presence of IL-4 DCs is not due to insufficient IL-23 and that IL-4 has a direct and dominant effect over IL-23 on the production of IL-17 by T cells. Indeed, IL-17 production by T cells in

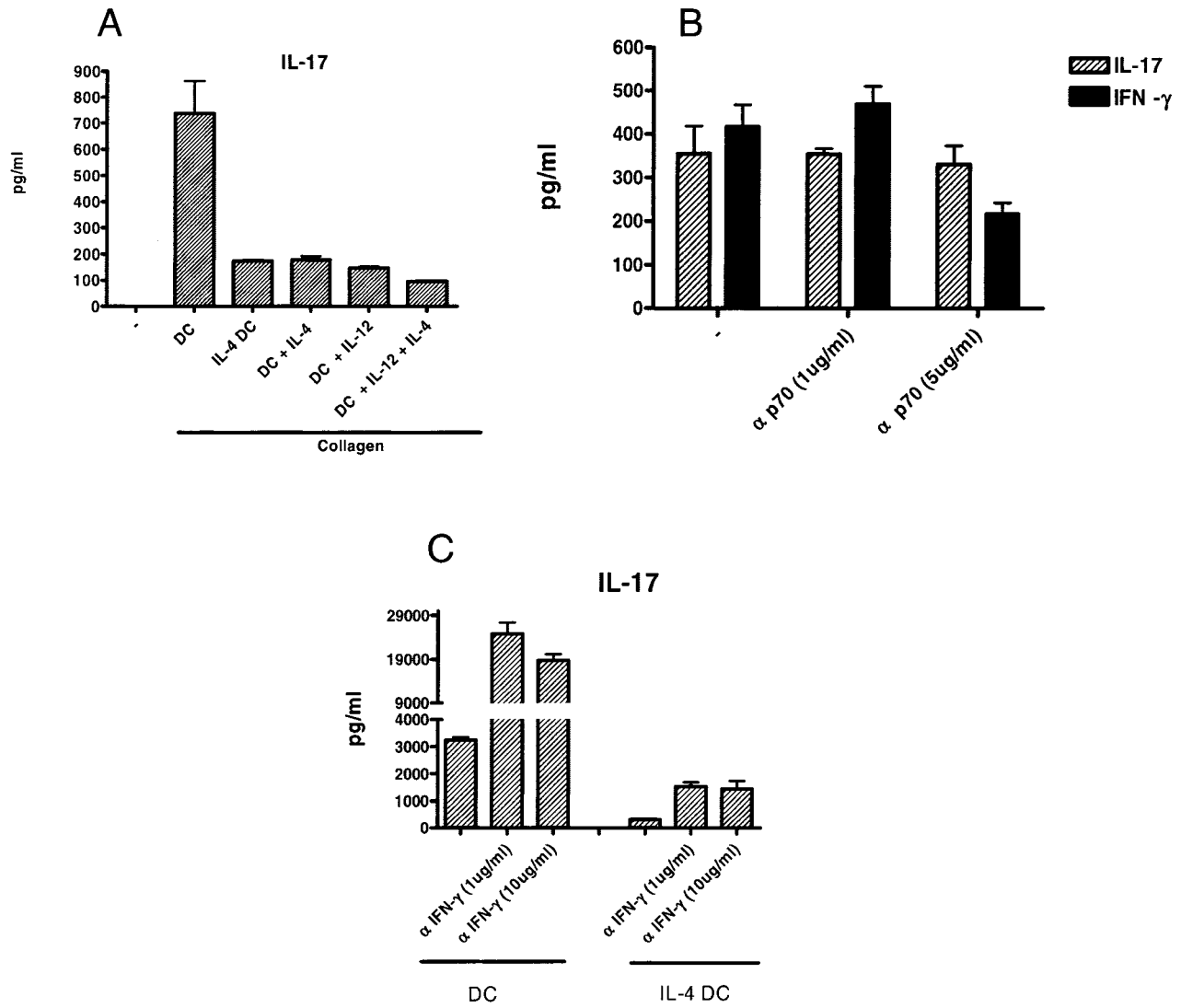


Figure 5. Suppression of interleukin-17 (IL-17) by IL-4 dendritic cells (DCs) in an IL-12- and interferon- γ (IFN γ)-independent manner. Splenic T cells were prepared from collagen-immunized mice and cultured with untransduced dendritic cells (DCs) or IL-4 DCs and collagen. **A**, IL-4 (10 ng/ml), and/or IL-12 (1 ng/ml) was added at the initiation of culture. Supernatants were collected after 5 days of culture and analyzed for IL-17 by enzyme-linked immunosorbent assay (ELISA). **B**, Neutralizing antibody to IL-12 p70 (α p70; 1 μ g/ml or 5 μ g/ml) was added to cultures of T cells with collagen and IL-4 DCs. Supernatants were collected after 5 days of culture and analyzed for IL-17 and IFN γ by ELISA. **C**, Neutralizing antibody to IFN γ (α IFN- γ ; 1 μ g/ml or 10 μ g/ml) was added to cultures of T cells with collagen and DCs or IL-4 DCs. Supernatants were collected after 5 days of culture and analyzed for IL-17 by ELISA. Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 3 independent experiments.

the presence of IL-4 DCs and neutralizing antibody to IL-4 was greater than that observed when T cells were cultured with untransduced DCs. We also found that the augmented IL-17 response seen after treatment with neutralizing IL-4 was only partly suppressed by blocking IL-23. It is possible that costimulatory molecule interactions, such as CD28/CD80-CD86 and inducible costimu-

lator (ICOS)/ICOSL, may be supporting IL-17 production in this setting.

We confirmed our data shown in Figure 4A by using untransduced DCs instead of IL-4 DCs. As shown in Figure 4B, suppression of IL-17 was evident when T cells were cultured with untransduced DCs and IL-4. IL-23 has been viewed as a primary driver of the IL-17

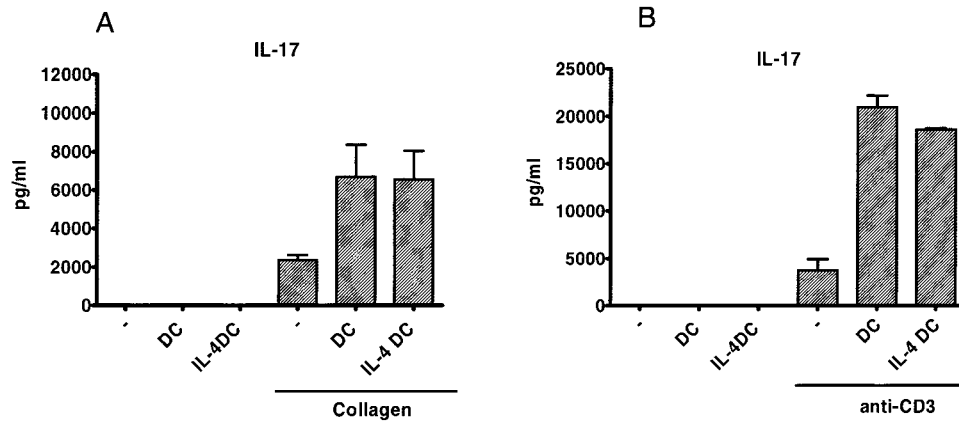


Figure 6. Lack of effect of interleukin-4 (IL-4) dendritic cells (DCs) on IL-17 production by T lymphocytes from mice with end-stage arthritis. Arthritis was induced in mice by intradermal injection of collagen and Freund's complete adjuvant. Mice with arthritis (scores of at least 3 or 4 in 1 paw) were killed on day 56 after collagen injection. T lymphocytes were prepared from spleens and cultured in vitro with DCs or IL-4 DCs and either **A**, collagen or **B**, anti-CD3. Supernatants were collected after 3 days (anti-CD3) or 4 days (collagen stimulation) of culture and analyzed for IL-17 by enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assays were performed in triplicate. Values are the mean and SEM. Results are from 1 experiment and are representative of 3 independent experiments.

response (6,18), and we found that adding IL-23 increased the production of IL-17, but when IL-4 and IL-23 were added together, there was no increase in IL-17 production. This effect was seen even at a very high concentration of IL-23 (100 ng/ml). Thus, IL-4 renders the T cells resistant to the IL-23-mediated production of IL-17.

To further study the role of IL-23 in this system, we set up cultures of T cells with untransduced DCs and used antibodies to the p19 subunit of IL-23. We found only a slight decrease in IL-17 production after neutralization of IL-23 (Figure 4C). It is possible that cell surface costimulatory ligands and/or proinflammatory cytokines other than IL-23 that are produced by DCs contribute to IL-17 production by T cells in a collagen rechallenge response.

Role of IL-12 and IFN γ in IL-4 DC-mediated suppression of IL-17 production by T cells. IL-23 and IL-12 share a common p40 subunit as well as a common receptor subunit (30). IL-12 is a potent inducer of IFN γ (19,30), and it has recently been shown that inhibition of IFN γ can enhance the development of IL-17-producing T cells (28). IL-4 DCs produce increased quantities of IL-12 and IFN γ , as compared with untransduced DCs, after stimulation with CD40L and lipopolysaccharide (25) (Figure 1A). We therefore evaluated the role of IL-12 and IFN γ in our system.

Splenic T cells from mice immunized with collagen were cultured with collagen and DCs for 5 days

(Figure 5A). IL-12 (1 ng/ml) and/or IL-4 (10 ng/ml) or anti-IL-4 (10 μ g/ml) was added at the initiation of culture. Culture supernatants were analyzed for IL-17 and IFN γ by ELISA. The presence of IL-12 suppressed IL-17 production (Figure 5A) and increased IFN γ production (data not shown). The effects of IL-12 and IL-4 on IL-17 were comparable. There was a modest increase in IL-17 production after neutralization of IL-4 in cultures with untransduced DCs (data not shown), which suggests that there were small amounts of endogenous IL-4 in these cultures. We then neutralized IL-12 in cultures of IL-4 DCs and T cells with neutralizing antibodies to IL-12 p70 (Figure 5B). Neutralizing IL-12 had no effect on IL-17 production in the presence of IL-4 DCs. As expected, neutralization of IL-12 resulted in decreased IFN γ production in these cultures. This suggests that although IL-12 can suppress IL-17, and IL-4 DCs produce increased quantities of IL-12, suppression of IL-17 production by IL-4 DCs is due to IL-4 and not IL-12.

IL-4 DCs produce increased quantities of IFN γ , and IFN γ suppresses IL-17. To study the role of IFN γ in IL-4 DC-mediated suppression of IL-17, T cells were cultured with DCs or IL-4 DCs and neutralizing antibody to IFN γ (Figure 5C). As expected, neutralizing IFN γ in cultures of T cells and DCs increased IL-17 production. Neutralizing IFN γ in cultures of T cells and IL-4 DCs led to only a modest increase in IL-17, less than that in cultures of T cells and DCs. This suggests

that while IFN γ produced by IL-4 DCs could account for some of the suppression, IL-4 plays a major and dominant role in suppression of IL-17 responses.

Effect of IL-4 DCs on IL-17 production by T cells from mice with established arthritis. We have shown that IL-4 DCs can modulate IL-17 production by collagen-specific T cells during the early stages of CIA. We also wanted to determine whether IL-4 DCs could alter IL-17 production by T cells from mice with established arthritis. Mice were immunized with collagen and CFA and were monitored for arthritis as described in Materials and Methods. Mice began to show signs of arthritis on day 28, and 70% of the mice had scores of 3 in at least 1 paw. On day 56 after collagen immunization, splenic T lymphocytes from mice with an arthritis score of 3 in at least 1 paw were obtained and purified. Mice that did not have arthritis and those that had maximum scores of 1–2 were excluded. T cells were then cultured with DCs or IL-4 DCs in the presence of collagen (Figure 6A) or the polyclonal stimulus anti-CD3 (Figure 6B), and supernatants were collected for measurement of IL-17 by ELISA. T cells from mice with late-stage CIA produced similar quantities of IL-17 in the presence and absence of IL-4 DCs, in either a polyclonal or a collagen-specific response. It is possible that the phenotype of T cells that secrete IL-17 is stabilized by the time the mice develop end-stage arthritis and is thus not susceptible to reduction by IL-4 DCs.

DISCUSSION

The Th1/Th2 paradigm has recently been modified to include a third type of differentiated T helper cell, called Th17, which secretes IL-17. Th17 cells have a distinct lineage and do not share a developmental pathway with Th1 or Th2 cells (28,29). IL-17 receptor is ubiquitously expressed (1). IL-17 stimulates the production of IL-1 β , TNF, IL-6, IL-8, G-CSF, and PGE₂ from macrophages and synoviocytes (2,5,31).

IL-23 augments IL-17 production by memory Th17 cells, but is itself not sufficient to generate Th17 cells. Data from transgenic mice expressing a T cell receptor for ovalbumin have shown that neutralization of IFN γ and IL-4, in addition to the presence of IL-23, enhances the generation of Th17 cells from naive CD4 T cells *in vitro*. Once the Th17 phenotype is stabilized, neutralization of IFN γ or IL-4 does not alter the IL-17 response (28). Th17 cells develop independently of STAT-1, STAT-4, and STAT-6 signaling and the transcription factors T-bet and c-Maf (28,29). Experiments done with CD28- or ICOS-knockout mice have shown

that these costimulatory molecules also play a role in the generation of Th17 cells (29). Recent studies have shown that IL-6, in combination with transforming growth factor β (TGF β), inhibits the generation of FoxP3-expressing T regulatory cells and induces the generation of Th17 cells (32–34). TGF β is not only critical to the development of Th17 cells, but it also induces the expression of IL-23 receptor, which facilitates Th17 survival and expansion (34). These studies provide evidence that Th1, Th2, and Th17 cells develop from naive T cells and that the generation of T regulatory cells and Th17 cells occurs via alternative pathways, which are selected according to the presence or absence of IL-6.

IL-17 has been detected and has been proposed to be pathogenic in systemic sclerosis (35), systemic lupus erythematosus (36), and RA (10). Anti-IL-17 antibody was shown to reduce the incidence and severity of experimental autoimmune encephalomyelitis, even though the splenic and lymph node cells obtained from the mice retained the capacity to produce IL-17. There was a lack of recruitment of Th17 cells into the brain secondary to a reduced expression of CCL2, CCL17, and CXCL1, suggesting that IL-17 can increase the expression of several lymphotactic chemokines. Transgenic mice that overexpressed IL-17 in the lung epithelium also had increased levels of several chemokines (29).

Neutralization of IL-17 has been shown to suppress the onset, and decrease the severity, of arthritis in animal models of RA (13,14,37). IL-17^{-/-} mice have reduced collagen-specific T cell responses during the initiation phase of arthritis and a reduced incidence and intensity of arthritis (15). Overexpression of IL-4 has been shown to down-regulate IL-17 and prevent cartilage destruction and bone erosion (38,39).

Investigators in our laboratory have previously shown that when bone marrow-derived DCs that overexpress IL-4 are injected into mice during the initiation phase of CIA, the incidence and severity of arthritis are reduced and that IL-4 DCs differentially regulate the production of IL-12/IL-23 after stimulation through their CD40 receptors. Since IL-17-knockout mice have a reduced incidence of arthritis and since local overexpression of IL-4 down-regulates IL-17 expression in the joints, we hypothesized that IL-4 DCs may be reducing the incidence of arthritis by decreasing the production of IL-17 by collagen-specific T cells. To study this, we isolated splenic T lymphocytes from DBA mice 15 days after collagen immunization and cultured them in the presence of untransduced or IL-4-transduced DCs in a collagen rechallenge response. IL-17 was measured in

the culture supernatants by ELISA or ELISpot. Our data showed that T cells cultured in the presence of IL-4 DCs produced less IL-17 and similar quantities of IFN γ in a collagen-specific response. This suggests that IL-17 is a cytokine of paramount importance in the early response to collagen and that IL-4 DCs reduce the incidence and severity of arthritis by decreasing the IL-17 response in T cells, even when the IFN γ response is fixed. It is likely that this muted IL-17 response during the early stages of arthritis translates into a reduced incidence and severity of arthritis. A decrease in both IL-17 and IFN γ was only seen when the polyclonal stimulus anti-CD3 was used. These effects were not limited to splenic T cells, but were also seen in lymphocytes from draining lymph nodes.

IL-4 DCs produce excessive IL-4, and we evaluated the effect of neutralizing IL-4 in cultures of IL-4 DCs or adding IL-4 to cultures with untransduced DCs. Our results showed that IL-4 can decrease the production of IL-17 by T cells cultured with DCs and collagen. Recent studies by Harrington et al (28) and Park et al (29), which were published when the experiments presented herein were near completion, have also shown that IL-4 can influence the production of IL-17, in that neutralizing IL-4 can lead to increased levels of IL-17. In our studies, neutralizing IL-4 in T cells cultured with IL-4 DCs resulted in reconstitution of the abrogated IL-17 response to collagen.

IL-23 has been implicated as a primary driver of IL-17 production by memory Th17 cells (17,18). We hypothesized that IL-4 may be reducing the production of IL-17 through an IL-23-dependent mechanism, possibly by down-regulating the production of IL-23 by DCs. If this were the case, then adding recombinant IL-23 would increase the production of IL-17. However, we found that IL-23 at 10 ng/ml did not alter IL-17 levels and that IL-23 at 100 ng/ml only modestly increased IL-17 production in the presence of IL-4 DCs. This amount of IL-23 is several hundred times higher than the amount of IL-4 present in the cultures. When the same amount of IL-23 was added to T cells cultured with untransduced DCs and collagen, we observed a much higher production of IL-17. This suggests that the IL-4-mediated suppression of IL-17 is not due to suppression of IL-23 production by IL-4 DCs. The data also indicate that IL-4 renders the T cells resistant to IL-23, at least with respect to IL-17 production, and that this resistance is not overcome by excess concentrations of IL-23. Primed T cells cultured with untransduced DCs and collagen showed high levels of IL-17 production, which was suppressed by IL-4 and was not reversed by the

addition of IL-23. These results provide further proof of IL-23 resistance in the presence of IL-4 and suggest that down-regulation of IL-17 by IL-4-transduced DCs is mediated by IL-4 and not by the retroviral transduction of DCs.

Furthermore, the increase in IL-17 production after neutralization of IL-4 was only modestly suppressed by the addition of anti-p19 antibody, thus suggesting that T cell production of IL-17 is only partly mediated by IL-23 in T cells cultured with IL-4 DCs or with untransduced DCs. It is likely that cognate costimulatory molecules play a role in the production of IL-17 by primed T cells obtained during the early stage of CIA.

IL-12, which shares a common subunit with IL-23, drives IFN γ production in T cells (40). Neutralizing IFN γ has been shown to increase the generation of Th17 cells (28,29). IL-4 DCs produce increased amounts of IL-12 and IFN γ after stimulation with lipopolysaccharide or CD40L (25). We also evaluated the effect of IL-12 and IFN γ in our system. We found that IL-12 suppressed IL-17 production, but the effect of IL-4 DCs on IL-17 production was independent of IL-12, since the IL-17 response was not restored by blocking IL-12. Neutralizing IFN γ did not reconstitute the IL-17 response, which provides further evidence that the IL-4 DC-mediated suppression of IL-17 is robust and dominant over IFN γ and is not secondary to excess IFN γ that is produced by IL-4 DCs or by T cells influenced by IL-12.

The mechanism underlying the IL-4-mediated suppression of IL-17 is not known. However, it is robust and dominant over IL-23 and IFN γ . It is possible that IL-4 down-regulates the expression of IL-23 receptor or induces T regulatory cells, which then suppress IL-17 production. It is also possible that IL-4 blocks the costimulatory molecule-mediated production of IL-17 by down-regulating receptor/ligand expression or function. IL-4 binding to its receptor on T cells could block intracellular signaling events that would otherwise have led to the production of IL-17.

T cells from mice with end-stage arthritis failed to decrease their production of IL-17 when cultured in the presence of IL-4 DCs. This suggests that while IL-4 DCs modulate the early initiation phase of arthritis by decreasing IL-17 production by T cells, they are unable to elicit such a response during the late phase of arthritis. It has been shown that IL-4 DCs can suppress CIA early after its clinical onset (41). Our data seem not to be in complete agreement with those obtained in previous studies by Lubberts et al (39), who reported that local injection of a viral vector expressing IL-4 resulted in the

amelioration of established inflammation and the reduction of IL-17 in the injected joint. It is possible, however, that the systemic IL-17 response in mice with established arthritis is regulated differently by IL-4 than is the local expression of IL-17 in arthritic joints.

The cytokine imbalance in CIA is difficult to explain by the prevailing Th1/Th2 paradigm and, instead, is likely to involve other cytokines, especially IL-17. The data currently available suggest that the IL-17-producing cells remain sensitive to IL-4 DCs for a limited interval following the appearance of joint inflammation. It is possible that, while the IL-17 response during the early initiation phase of CIA is amenable to regulation by IL-4, the IL-17 response during end-stage arthritis is mediated by a distinct set of costimulatory signals that cannot be overcome by IL-4. Alternatively, the IL-17-producing T cells in end-stage arthritis are at a point in their differentiation at which IL-4 receptor signaling is defective.

Distinct windows appear to exist in the IL-17 response from T cells in arthritis, during which it can be modulated by changing the cytokine environment. Once the IL-17 response is stabilized, as in end-stage arthritis, then such changes are not possible or, at least, are more difficult to accomplish. This paradigm helps to explain the early events in immune-mediated arthritis and possible ways to modulate key pathogenic responses. Such an understanding will help in the development of novel therapies targeted to IL-17 in RA. Further studies evaluating the role of costimulatory molecules in the generation and regulation of IL-17-producing T cells, as well as the *in vivo* IL-17 response in CIA and its modulation by administration of IL-4 DCs, are ongoing.

AUTHOR CONTRIBUTIONS

Dr. Fox had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Dr. Sarkar, Ms Tesmer, and Dr. Fox.

Acquisition of data. Dr. Sarkar, Ms Tesmer, Dr. Hindnavis, and Ms Endres.

Analysis and interpretation of data. Dr. Sarkar, Ms Tesmer, Dr. Hindnavis, Ms Endres, and Dr. Fox.

Manuscript preparation. Dr. Sarkar, Ms Tesmer, and Dr. Fox.

Statistical analysis. Dr. Sarkar.

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