

Inducible costimulator is essential for collagen-induced arthritis

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CD4⁺ helper Th cells play a major role in the pathogenesis of rheumatoid arthritis. Th cell activation, differentiation, and immune function are regulated by costimulatory molecules. Inducible costimulator (ICOS) is a novel costimulatory receptor expressed on activated T cells. We, as well as others, recently demonstrated its importance in Th2 cytokine expression and Ab class switching by B cells. In this study, we examined the role of ICOS in rheumatoid arthritis using a collagen-induced arthritis model. We found that ICOS knockout mice on the DBA/1 background were completely resistant to collagen-induced arthritis and exhibited absence of joint tissue inflammation. These mice, when immunized with collagen, exhibited reduced anti-collagen IgM Ab's in the initial stage and IgG2a Ab's at the effector phase of collagen-induced arthritis. Furthermore, ICOS regulates the *in vitro* and *in vivo* expression of IL-17, a proinflammatory cytokine implicated in rheumatoid arthritis. These data indicate that ICOS is essential for collagen-induced arthritis and may suggest novel means for treating patients with rheumatoid arthritis.

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Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by autoantibody-mediated chronic inflammation of multiple joints and destruction of cartilage and bone (1). Collagen-induced arthritis (CIA) is the most widely used mouse model for RA (2, 3), in which immunization of genetically susceptible strains of mice, such as DBA/1, with type II collagen (CII) leads to the development of a severe polyarticular arthritis mediated by immune mechanisms. The autoimmune response in the CIA animal model involves CII-specific T cells and B cells and their products, proinflammatory cytokines, and anti-CII Ab's. There are considerable data to implicate CII-reactive CD4⁺ cells as primary mediators of disease induction and autoantibody production by B cells as a major immune mechanism leading to the localized chronic inflammatory response (4–6). In addition to providing helper cytokines such as IFN- γ to mediate Ab production by B cells, T cells but not B cells are often found to infiltrate into the joint tissues and, per-

haps by producing proinflammatory cytokines, cause inflammatory reactions that involve macrophages, neutrophils, and mast cells (7). T cell cytokines such as IL-17 have been found in the synovium of arthritis patients or CIA mice (8, 9), although the local regulation of their expression has not been understood.

Th cell activation, differentiation, and immune function are regulated by costimulatory molecules. CD28 is the most important costimulatory receptor on T cells. It binds to B7.1 (CD80) and B7.2 (CD86) on activated APCs. Studies using T cells derived from CD28-deficient mice demonstrated its role in T cell activation (10). Consistent with this, CD28 knockout mice on the DBA/1 background are resistant to CIA (11). The function of CD28, however, in the effector phase is less dominant. This is probably due to the induction of its antagonist, CTLA-4, on activated T cells, which binds the same ligands with much higher affinity and functions to inhibit T cell proliferation and cytokine expression (12).

Inducible costimulator (ICOS) is the third member of the CD28 family. Not expressed by naive Th cells, it is induced after T cell activation (13, 14). The ligand for ICOS, B7H (also named B7RP-1) is constitutively expressed on B cells and in nonlymphoid tissues such as the lung (13, 15). Most interestingly, B7H expression is strongly induced in fibroblasts by TNF- α and in several nonlymphoid tissues after LPS injection in mice (15). Recently, we, as well as others, constructed and analyzed mice deficient in the ICOS gene (16–18). ICOS-deficient mice exhibited impaired Ig class-switching and germinal center reactions (16, 17). On the other hand, we found that ICOS^{-/-} mice were extremely sen-

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Nonstandard abbreviations used: rheumatoid arthritis (RA); collagen-induced arthritis (CIA); chicken type II collagen (CII); inducible costimulator (ICOS); prostaglandin E₂ (PGE₂).

sitive to experimental autoimmune encephalomyelitis (EAE) (16). Therefore, ICOS appears to play opposite roles in humoral and inflammatory responses. The function of ICOS in Ab-mediated autoimmune diseases such as RA has not been addressed, however.

In this paper, we have analyzed the role of ICOS in RA using the CIA model. We found that ICOS knockout mice on the DBA/1 background were completely resistant to CIA. Compared with *ICOS*^{+/+} mice, *ICOS*^{-/-} exhibited reduced anti-collagen IgM and IgG2a titers, indicating that ICOS regulates to some extent the humoral responses. Furthermore, ICOS regulates the expression of IL-17 in vitro and in vivo. Therefore, ICOS plays an important role in the pathogenesis of CIA; it therefore provides an important and novel target for therapy of RA patients.

Methods

Mice. ICOS knockout mice were backcrossed six generations onto the DBA/1 background. *ICOS*^{+/+} and *ICOS*^{-/-} mice, all on F₆ backcross, were interbred, and their offspring were used for the experiments. All mice were 8–10 weeks old at the time of immunization. The mice were housed in a specific pathogen-free facility.

Induction of collagen-induced arthritis. Arthritis was induced and scored in male *ICOS*^{+/+} and *ICOS*^{-/-} mice by immunizing with CII (Sigma-Aldrich, St. Louis, Missouri, USA) (19). Chicken CII was dissolved in cold 10 mM acetic acid overnight at 4°C and emulsified with equal volume of CFA (Sigma-Aldrich) to give a final concentration 1 mg/ml. Experimental mice were immunized intradermally at the base of the tail with 100 µl of emulsified chicken CII on day 0 and boosted with the same agent on day 21 and 42. Severity of disease was evaluated by visual inspection of the paws. Each paw was scored for the degree of inflammation on a scale from 0 to 4: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; 2, erythema and mild swelling extending from ankle to the midfoot; 3, erythema and mild swelling extending from ankle to metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot, and digits. Scores from all four paws were added to give the total for each animal. The experiments were performed in accordance with the regulation of the Animal Care Committee of University of Washington.

Histopathological analyses. Experimental mice were euthanized by CO₂ inhalation. Routine necropsy was performed, and the distal appendages were placed in 10% neutral-buffered formalin for 7 days. The tissue sections were processed for routine paraffin embedding, and hematoxylin and eosin staining following decalcification.

Measurement of anti-CII Ab's. Anti-collagen Ab (IgM, IgG1, IgG2a) production was measured in the serum of mice either 14 days after the first immunization or at the end of the experiment (day 58) by ELISA. Briefly, serum samples from immunized mice were added in a threefold serial dilution on plates precoated with 10

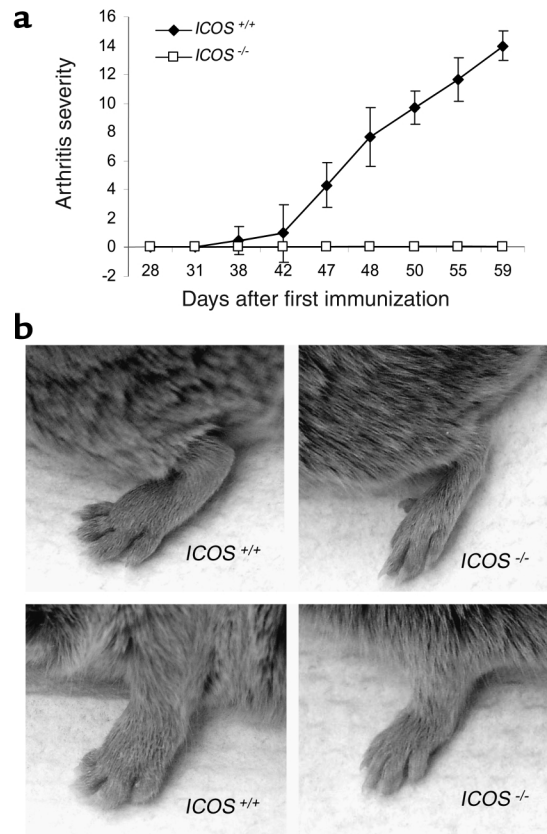


Figure 1

Resistance to collagen-induced arthritis by ICOS-deficient mice. *ICOS*^{-/-} and *ICOS*^{+/+} mice on DBA/1 background were immunized at the base of the tail on days 0, 21, and 42 with type II chicken collagen in CFA. (a) Disease severity was scored on days 38, 42, 47, 48, 50, 55, and 58 by visual inspection of mouse paws. Each paw was scored for the degree of inflammation on a scale 0–4 as indicated in Methods. Scores were then added together. The data shown are a representative of two experiments on a total of more than ten mice in each group, with similar results. (b) Representative photos of forepaws and hindpaws of *ICOS*^{+/+} and *ICOS*^{-/-} mice 58 days after first immunization with chicken CII.

µg/ml chicken CII. Collagen-specific Ab's were detected with biotinylated goat anti-mouse IgM and rat anti-mouse IgG1 and IgG2a Ab's (Southern Biotechnology Associates, Birmingham, Alabama, USA).

T cell cytokine measurement. Spleen cells from *ICOS*^{+/+} and *ICOS*^{-/-} mice 14 days after the first immunization with chicken CII were either unstimulated or stimulated with 50 or 100 µg chicken CII, and cytokine production (IFN-γ, TNF-α, IL-4, and IL-17) in the 3-day culture supernatant was determined by ELISA (PharMingen, San Diego, California, USA). Proliferation was assayed after 3 days of treatment with different doses of chicken CII by adding ³H-thymidine to the culture for the last 8 hours. To further test the role of ICOS in costimulation of DBA/1 T cells, naive CD4⁺ T cells from lymph nodes and spleens of 6- to 8-week-old wild-type DBA/1 mice were isolated, as we reported previously (16). Purified naive CD4⁺ T cells were stimulat-

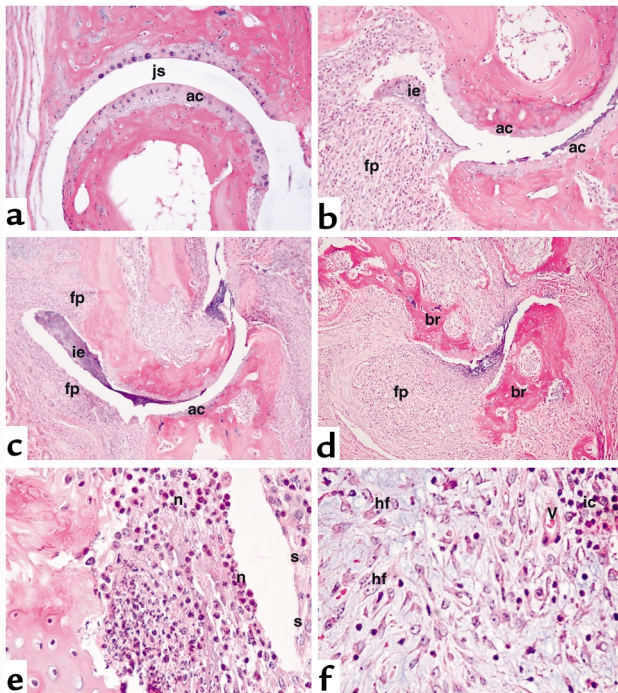


Figure 2

Representative hematoxylin and eosin–stained sections of intraphalangeal joints from *ICOS*^{+/+} and *ICOS*^{-/-} mice. (a) *ICOS*^{+/+}. Notice the even and clear joint space (js) and smooth articular cartilage (ac). Original magnification $\times 200$. (b–f) *ICOS*^{-/-}. (b) Notice the fibrovascular synovial and periarticular proliferation (fp) erosion of articular cartilage (ac) intra-articular exudates (ie) and osteolysis in severely affected joints (d). Original magnification $\times 200$. (c) Original magnification $\times 100$. (d) Articular bone remnants (br) from a severely affected mouse. Original magnification $\times 100$. (e) Intra-articular exudates composed of neutrophils (n), other inflammatory cells, and hyperplastic synoviocytes (s) admixed within cellular debris. Original magnification $\times 400$. (f) High magnification of the fibrovascular proliferative tissues composed of primarily hyperplastic fibrocytes (hf), neovascularization (v), and inflammatory cells (ic). Original magnification $\times 600$.

ed in triplicate with plate-bound anti-CD3 in the absence or presence of anti-CD28 and/or anti-ICOS. On day 4, Th cytokine production in the supernatant was measured by ELISA (PharMingen). To examine CD40L expression, lymph node cells from *ICOS*^{+/+} and *ICOS*^{-/-} mice were activated with α CD3 (3 μ g/ml) and then stained with a biotinylated α CD40L Ab, followed by streptavidin–R-phycoerythrin (Southern Biotechnology Associates) for flow-cytometry analysis.

Results

RA is an autoimmune disease affecting a large percentage of the human population. CD4⁺ T cells play crucial roles in the pathogenesis of the disease. How their function is regulated, however, is still poorly understood. In the current study, we examined the role of ICOS costimulatory receptor in CIA.

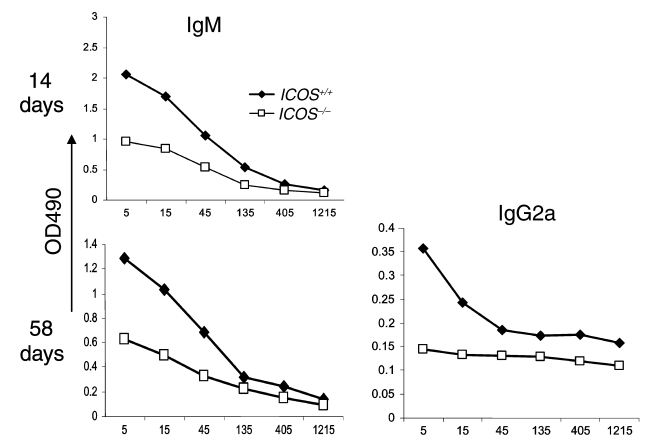
ICOS knockout mice are completely resistant to CIA. We analyzed the role of ICOS in RA using the murine CIA model. We backcrossed ICOS knockout mice six generations onto the DBA/1 background. *ICOS*^{-/-} and *ICOS*^{+/+} DBA/1 mice were immunized at the base of the

tail on day 0, 21, and 42 with CII in CFA. *ICOS*^{+/+} mice developed severe joint inflammation as seen by others, evidenced by marked swelling and erythema of the hindpaws and forepaws (Figure 1a). Inflammation in these paws included the wrist and ankle and extended distally through the limb and digits (Figure 1b). In contrast, *ICOS*^{-/-} DBA/1 mice were uniformly resistant to CIA (Figure 1a) and had no sign of paw or joint swelling (Figure 1b).

We further examined the experimental mice histologically. Only the *ICOS*^{+/+} DBA/1 mice with clinical disease had histopathological lesions consistent with collagen-induced arthritis. We examined the pedal joints of *ICOS*^{+/+} DBA/1 mice and *ICOS*^{-/-} DBA/1 mice (Figure 2). All of the *ICOS*^{-/-} DBA/1 joints were histologically normal with no sign of tissue degeneration and inflammation. All of the *ICOS*^{+/+} DBA/1 mice had severe histopathological lesions characterized by extensive fibrovascular and proliferative synovitis composed of abundant fibroblasts, hypertrophic synoviocytes, and marked infiltration of inflammatory cells, primarily neutrophils and macrophages, lesser lymphocytes, and mast cells (Figure 2, b–f), which extended into the joint space. Additionally, in severely affected joints, there was moderate to severe cartilage destruction, and marked remodeling of bone. Often, the fibrovascular

Figure 3

Immunized *ICOS*^{-/-} mice exhibited greatly reduced levels of anti-collagen IgM and IgG2a compared to *ICOS*^{+/+} mice. Anti-collagen Ab's (IgM and IgG2a) were measured in the sera by ELISA. The sera from *ICOS*^{+/+} and *ICOS*^{-/-} mice (three mice in each group), either 14 days after the first immunization or at the end of the experiment (day 58), were subjected to a threefold serial dilution; the concentrations of collagen-specific IgM and Ig2a were analyzed by ELISA, and average OD reading was indicated.



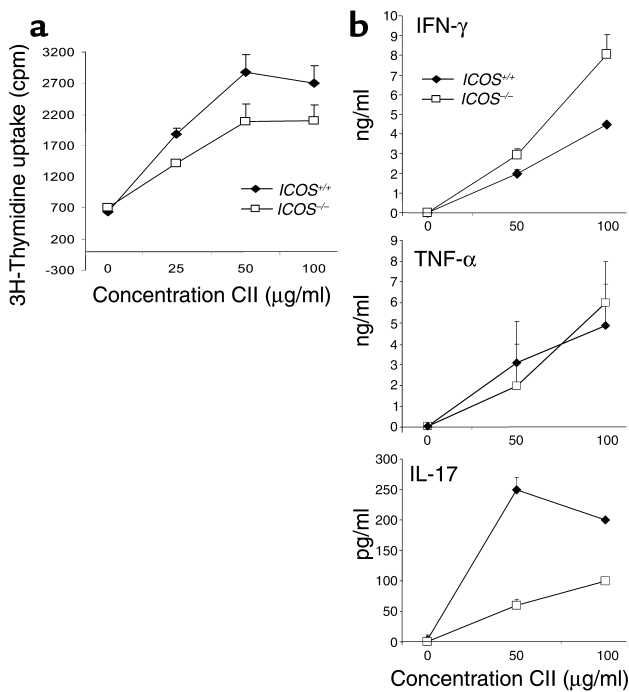


Figure 4 Reduced proliferation and IL-17 expression by *ICOS*^{-/-} T cells after CII immunization. *ICOS*^{+/+} and *ICOS*^{-/-} mice were immunized with chicken CII in CFA. On day 14, spleen cells were isolated. (a) For the proliferation assay, spleen cells were treated with 25, 50, 100 µg/ml chicken CII for 3 days. ³H-thymidine incorporation during the last 8 hours was measured. (b) Cytokine production (IFN-γ, TNF-α, and IL-17) in supernatant of spleen cells activated with 50 or 100 µg/ml chicken CII for 3 days was measured by ELISA.

proliferation and inflammation extended into the peri-articular connective tissues and adjacent musculature.

Reduced anti-CII humoral responses in the absence of ICOS. To better characterize the immune mechanisms underlying CIA resistance by *ICOS*^{-/-} mice, we measured first anti-collagen Ab production (IgM, IgG1, IgG2a) in the serum of mice at the end of the experiments (day 58) (Figure 3). CIA is best characterized by pathogenic anti-CII IgG, especially IgG2a Ab's. Consistent with the resistance of *ICOS*^{-/-} mice to CIA, we found they exhibited greatly reduced levels of IgG2a Ab to anti-CII. On the other hand, low levels of anti-CII IgG1 production were not severely affected in these mice (data not shown). Interestingly, anti-CII IgM was also reduced in the absence of ICOS.

ICOS has been shown previously by others and us to play an important role in regulating B cell Ig class switching (16–18, 20). We therefore examined only if class-switching was affected in the absence of ICOS. Serum anti-CII Ab's were measured from *ICOS*^{+/+} and *ICOS*^{-/-} mice 14 days after the first immunization. At this time point, there was little anti-collagen IgG1 and IgG2a production by both groups (data not shown). On the other hand, IgM was produced most significantly by *ICOS*^{+/+} mice (Figure 3). Surprisingly, we found at this stage that *ICOS*^{-/-} DBA/1 already exhibit-

ed greatly reduced anti-CII IgM in serum (Figure 3). This result suggests an important role of ICOS in the initial B cell response, which can result in IgG2a deficiency and CIA resistance by *ICOS*^{-/-} mice.

T cell responses in the immunized *ICOS*^{-/-} mice. We showed previously that ICOS is important for T cell activation and function (16). Defective B cell response in *ICOS*^{-/-} could thus be caused by insufficient T cell priming or lack of certain T cell cytokine products. To analyze antigen-specific T cell responses in *ICOS*^{+/+} and *ICOS*^{-/-} mice, we immunized *ICOS*^{+/+} and *ICOS*^{-/-} with CII, and their spleen cells harvested 14 days later were restimulated with different doses of CII. T cell proliferation and cytokine secretion were examined. *ICOS*^{-/-} cells exhibited moderately reduced T cell proliferation, suggestive of a minor role of ICOS in T cell priming in vivo (Figure 4a).

Since the marginally reduced T cell priming described above could not fully explain the complete resistance of *ICOS*^{-/-} mice to CIA, we further examined IFN-γ, TNF-α, IL-4, and IL-17 production by spleen cells in response to CII (Figure 4b). No IL-4 could be detected by either *ICOS*^{+/+} or *ICOS*^{-/-} cells, correlating with the notion that the CIA protocol generates a Th1 response in vivo. No difference was observed in expression of IFN-γ in *ICOS*^{-/-} and *ICOS*^{+/+} mice. Normal IFN-γ production by *ICOS*^{-/-} mice would also argue against a role of ICOS in regulating Ig class switching to IgG2a. In addition, TNF-α, an important cytokine for RA, was found to be produced normally in the absence of ICOS. Therefore, a TNF-α-independent mechanism must confer CIA resistance in the knock-

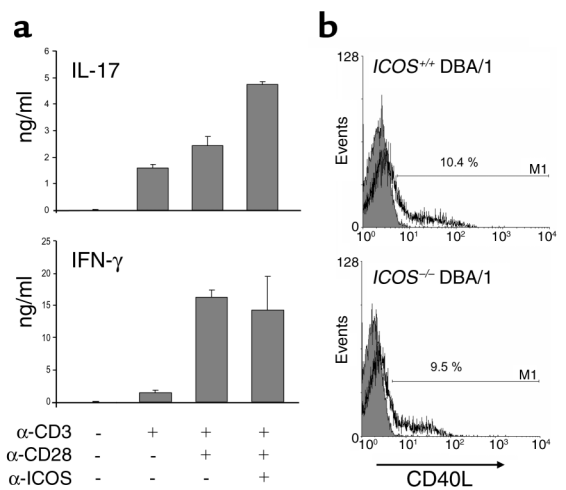


Figure 5 ICOS regulates IL-17 but not CD40L expression. (a) Naive Th cells from *ICOS*^{+/+} mice were stimulated with the indicated combination of anti-CD3, anti-CD28, and anti-ICOS Ab's. After 4 days, the amounts of Th cytokines (IFN-γ and IL-17) were measured by ELISA. (b) Lymph node cells from *ICOS*^{+/+} and *ICOS*^{-/-} mice were activated with αCD3 for 6 hours, stained with mAb to CD40L plus streptavidin-phycoerythrin, and analyzed by flow cytometry (open curves). Control staining is shown as dark curves. The data represent one of two independent experiments with the same results.

out mice, suggesting that an anti-ICOS therapy for RA may serve as an alternative for patients who fail TNF- α -targeted therapy.

ICOS regulates IL-17 expression. Interestingly, spleen cells from *ICOS*^{-/-} mice produced a remarkably decreased amount of IL-17 in response to CII (Figure 4b). IL-17 is a new cytokine secreted by CD4⁺-activated memory T cells and is frequently found in RA synovium (8, 21, 22). The cellular responses to IL-17 are similar to IL-1, including IL-6, IL-8, G-CSF, and prostaglandin E₂ (PGE₂) production (8, 21). The effect of IL-17 on cartilage was associated with its destruction, including activation of NO, and IL-17 has also been described as a potent stimulator of osteoclastic bone reabsorption due to PGE₂ synthesis (23–25). IL-17 produced by CD4⁺ cells in the joint tissue may contribute to the inflammatory process involved in RA. In support of this notion, overexpression of IL-17 in the joints resulted in inflammatory infiltration of the synovium and aggressive cartilage degradation (26). Blockade of IL-17 action with a soluble receptor significantly reduced the CIA, including a clear suppression of joint damage (27). Thus, defective IL-17 production in the *ICOS*^{-/-} mice may contribute to lack of joint inflammation in these mice. Since ICOS ligand, B7H, is expressed in inflamed tissues, ICOS-B7H interaction may regulate IL-17 local expression in the joint tissue in the CIA model and mediate the inflammatory responses. On the other hand, a recent study of the IL-17 knockout mice indicates a novel role for IL-17 in humoral immune responses (28). Although the role of IL-17 in autoantibody production in the CIA model has not been formally addressed, the reduced anti-CII autoantibodies in *ICOS*^{-/-} mice possibly could be attributed to the IL-17 deficiency.

IL-17 has emerged as an important T cell-derived cytokine in many aspects of immune responses; however, how it is regulated is very poorly understood. Our result indicates a novel role of ICOS in IL-17 expression in vivo. To further examine ICOS regulation of IL-17 expression, we tested the ability of ICOS costimulation to induce this cytokine production by naive T cells. C398-4A is a mAb against ICOS (29, 30); its specificity was further confirmed by us previously based on its lack of binding to *ICOS*^{-/-} cells (16). We stimulated naive Th cells from DBA/1 mice with different combinations of anti-CD3, CD28, and ICOS. CD28 costimulation greatly increased IFN- γ but not IL-17 expression. Since ICOS upregulation on activated T cells require CD28 costimulation (31), we used a combination of anti-CD3, CD28, and ICOS to examine ICOS effect. We did not find any enhancement of IFN- γ production by this treatment as compared with cells treated only with anti-CD3 and CD28 (Figure 5a). On the other hand, ICOS costimulation significantly increased IL-17 production by activated T cells. This result indicates a unique regulatory role for ICOS in IL-17 expression by activated T cells.

ICOS has been implicated previously in regulating CD40L expression by activated T cells (17). In addition to T cell cytokines, we also examined the CD40L expression. *ICOS*^{+/+} and *ICOS*^{-/-} lymph node cells were activated with anti-CD3 for 6 hours, and cell-surface CD40L expression was examined by flow cytometry. Consistent with our previous report (16), *ICOS*^{-/-} cells expressed CD40L at the same level as the *ICOS*^{+/+} cells. Therefore, it is unlikely that ICOS regulates CIA response through a CD40L-dependent mechanism.

In conclusion, we found *ICOS*^{-/-} mice on DBA/1 background to be completely resistant to CIA. *ICOS*^{-/-} mice exhibited reduced B cell response in anti-CII Ab production. In addition, we report a novel role of ICOS in regulation of IL-17 expression. Since IL-17 has been shown to mediate joint tissue inflammation and is important in humoral immune responses, ICOS thus possibly mediates autoimmune arthritis by regulating IL-17 expression. Considering that ICOS is only expressed on activated T cells, anti-ICOS approaches may therefore provide specific T cell-based therapy for RA patients.

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