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ICOS Mediates the Development of Insulin-Dependent Diabetes Mellitus in Nonobese Diabetic Mice¹

Daniel Hawiger,* Elise Tran,* Wei Du,[‡] Carmen J. Booth,[§] Li Wen,[‡] Chen Dong,[¶] and Richard A. Flavell^{2*†}

Initiation of diabetes in NOD mice can be mediated by the costimulatory signals received by T cells. The ICOS is found on Ag-experienced T cells where it acts as a potent regulator of T cell responses. To determine the function of ICOS in diabetes, we followed the course of autoimmune disease and examined T cells in ICOS-deficient NOD mice. The presence of ICOS was indispensable for the development of insulinitis and hyperglycemia in NOD mice. In T cells, the deletion of ICOS resulted in a decreased production of the Th1 cytokine IFN- γ , whereas the numbers of regulatory T cells remained unchanged. We conclude that ICOS is critically important for the induction of the autoimmune process that leads to diabetes. *The Journal of Immunology*, 2008, 180: 3140–3147.

Costimulation determines the function and fate of the T cells responding to antigenic challenge (1). The ICOS, a member of the CD28/CTLA-4 family, is expressed after T cell activation and is thought to determine the function of T cells. Stimulation by ICOS has been shown to enhance T cell proliferation and to influence the production of both Th1 and Th2 cytokines (2–9). Accordingly, ICOS signaling shows diverse effects on the experimental course of several autoimmune diseases. The blockade of ICOS ameliorates symptoms of collagen-induced arthritis, murine lupus nephritis, and transplant rejections (10–17). However, the absence of ICOS exacerbates the symptoms of autoimmune experimental acute encephalomyelitis (EAE)³ and inhibits the functions of regulatory T cells (Tregs) in experimental models of asthma and diabetes (5, 18, 19). In EAE, blockade of ICOS immediately after the induction of the disease exacerbates the encephalomyelitis, a result consistent with data obtained from mice with a targeted deletion of ICOS; however, a delayed blockade of ICOS was found to ameliorate the symptoms of EAE (5, 20, 21). This disparity of function further underscores the complex role that ICOS plays as a costimulatory molecule in T cells.

A majority of the experimental autoimmunity models in which a role of ICOS was examined relied on the active induction of the autoimmunity by immunizations with a model Ag. In other disease

models, such as murine lupus, where the role of ICOS has been studied, the symptoms of the autoimmune disease accumulate only later in life and are at least in part dependent on the prolonged production of autoantibodies and accumulation of immune complexes (11). In contrast, type 1 diabetes mellitus (T1DM) is a spontaneous disease caused by a T cell-driven, autoimmune destruction of the insulin-producing β cells in the pancreatic islets that begins in NOD mice already by a few weeks of age (22). The function of diabetogenic T cells can be influenced by the signals received through cell surface molecules belonging to several families of costimulators. Members of the CD28/CTLA-4 family of costimulatory molecules such as CD28, CTLA-4, and PD-1 can modulate the function of diabetogenic T cells to avert islet inflammation and the ensuing hyperglycemia, but the role of ICOS in the development of diabetes remains unclear (23–27). Although ICOS has been proposed to be involved in establishing homeostasis of Tregs acting to prevent insulinitis (19), the blocking of both ICOS and CD40L (CD154) had a synergistic effect of prolonging islets grafts survival and decreasing the incidence of diabetes in NOD mice. However, an Ab blockade of ICOS in combination with other treatments, such as cyclosporine, showed no effect on islet survival (14, 28, 29).

In this study, we report that genetic deletion of ICOS in NOD mice leads to complete protection from the T1DM with an accompanying amelioration of islet inflammation and decreased levels of autoantibodies in the serum. We further show that in T cells, the deletion of ICOS resulted in a decreased production of the Th1 cytokine IFN- γ whereas the numbers of CD25 and FoxP3 positive regulatory cells remained unchanged.

Materials and Methods

Mice

ICOS^{-/-} mice (5) on a mixed 129/B6 background were backcrossed 15 generations with NOD mice to generate ICOS^{-/-} NOD mice. Mice were genotyped by PCR for multiple Idd markers including D1Mit74, D1Mit178, and D1Mit180 that are specific for the Idd5 locus. PCR protocols were obtained from Type I diabetes resource center of The Jackson Laboratory and Whitehead Institute. Experimental animals were produced by breeding the ICOS^{-/-} with ICOS^{+/-} mice and equal numbers of age and sex matched ICOS^{-/-} and ICOS^{+/-} littermates were used for the experiments. All mice were maintained in our facility under specific pathogen-free conditions and used in accordance with the institutional guidelines.

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³ Abbreviations used in this paper: EAE, experimental acute encephalomyelitis; Treg, regulatory T cell; T1DM, type 1 diabetes mellitus; GAD, glutamic acid decarboxylase.

Assessment of diabetes development

Diabetes was monitored by measuring urine glucose level with Diastix (Bayer). Blood glucose was measured with One Touch test strips (LifeScan).

Histopathology

The mice were euthanized, and the pancreas was harvested and stained with H&E. All sections of the fixed pancreata were examined by light microscopy using a semiquantitative criterion-based methodology adapted from Ref. 30. Severity scores ranged from 0 to 5, and numerical values of 0 (within normal limits, absent), 1 (minimal), 2 (mild), 3 (moderate), 4 (marked), and 5 (severe) were assigned according to presence and severity of inflammation, and loss of islet cells.

Detection of islet autoantibody

Anti-insulin and anti-glutamic acid decarboxylase (GAD)65 autoantibodies were analyzed by ELISA. The plates were coated with recombinant human insulin (Lilly) or GAD65 (a gift from Dr. Peter van Endert, Institut National de la Santé et de la Recherche Médicale, France) blocked, and serum samples with different dilutions were incubated in the wells followed by incubation with alkaline phosphatase conjugated goat anti-mouse IgG and development with pNPP. For detection of total IgG, the AffiniPure and HRP-conjugated goat anti-mouse IgG from Jackson ImmunoResearch were used.

Flow cytometry and Abs used for staining

Anti-CD4 (L3T4), anti-IFN- γ (XMG1.2), anti-CD25 (PC61), anti-IL-17 (TC11-18H10), anti-IL-4 (11B11) were from BD Pharmingen. Anti-FoxP3 (FJK16s) and anti-ICOS (7E17G9) were from eBioscience. For intracellular cytokine staining, lymphocytes were stimulated *in vitro* for 4 h with PMA/ionomycin. Cells were fixed and permeabilized using Fixation/Permeabilization buffers from eBioscience according to the manufacturers' manual.

Production of Abs

Anti-B7h hybridomas were a gift from Dr. W. Sha (University of California Berkeley, Berkeley, CA) (31). Anti-mouse IgG were produced in a serum-free medium substituted with a Nutridoma-SP reagent (Roche) and affinity purified on an Agarose-protein G column.

Real-time RT-PCR analysis

RNA was isolated from the sorted peripheral CD4 T cells using TRIzol Reagent (Invitrogen Life Technologies) and a Qiagen mRNAeasy kit (Qiagen). Total RNA was reverse transcribed and the cDNA was subsequently used for quantitative PCR on an ABI Prism instrument using either commercial primer-probe sets (Mm00483137_m1 CD28, Mm00486849_m1 CTLA-4; Applied Biosystems) or published primer and probe sequences for mouse *ICTLA-4* and *sCTLA-4* (32) and IFN- γ (33). The results of Q-PCR were standardized to the hypoxanthine guanine phosphoribosyl-transferase expression levels (33).

Results

ICOS is required for the development of diabetes in NOD mice

Costimulation determines the function and fate of the T cells responding to antigenic challenge, and some costimulatory molecules such as CD28, CTLA-4, or PD-1 play a protective role in T1DM (23, 24, 26). To examine the role of ICOS in the development of T1DM, we developed ICOS-deficient NOD mice by crossing for 15 generations the original ICOS^{-/-} animals generated in our laboratory (5) with the NOD strain (see *Materials and Methods*). To examine the development of diabetes in ICOS-deficient mice, we monitored glucose levels in the ICOS^{-/-} and ICOS^{+/-} littermates (Fig. 1A). We found that ICOS^{-/-} NOD females (Fig. 1A) and males (data not shown) were completely resistant to the development of diabetes because none of the ICOS^{-/-} mice studied developed hyperglycemia when followed for >60 wk whereas the incidence of diabetes in ICOS^{+/-} was ~65% (35% remaining nondiabetic; Fig. 1A). The incidence in ICOS^{+/-} mice was similar to ~75% incidence observed in wild-type NOD control mice used to generate ICOS-deficient NOD mice (*Materials and Methods*

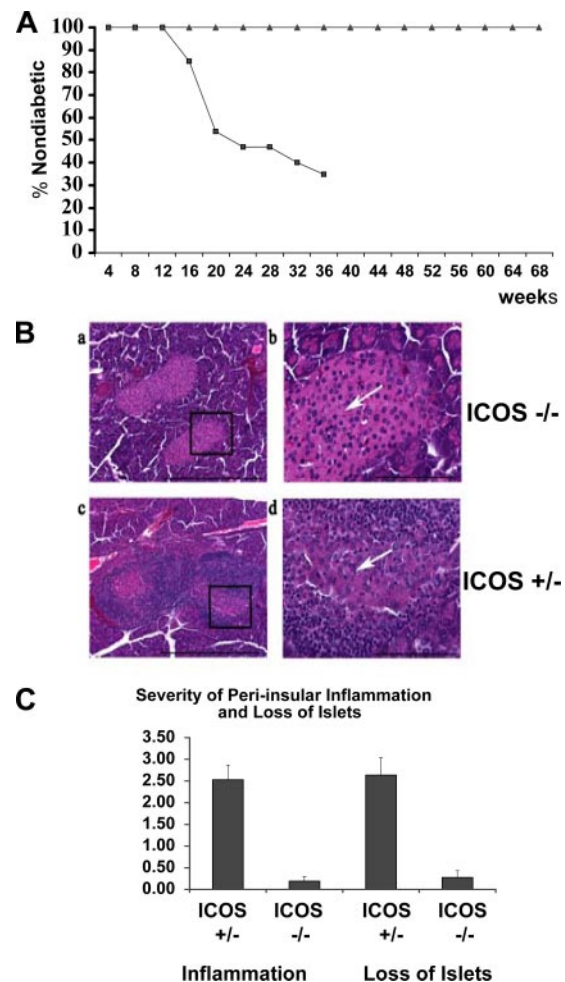


FIGURE 1. Protection from diabetes and absence of insulinitis in female ICOS^{-/-} NOD mice. **A**, ICOS^{-/-} mice were bred into NOD background for 15 generations. Urine glucose levels were monitored biweekly starting when mice reached 10 wk of age. The increased glucose urine levels were verified by measuring blood glucose content exceeding 250 mg/dl and blood glucose was in a range of 280–500 mg/dl in ICOS^{+/-} diabetic animals. Additionally, blood glucose levels were measured in all female ICOS^{-/-} littermates and were found to be in the range of 85–120 mg/dl. ICOS^{-/-} triangles, ICOS^{+/-} squares/ICOS^{-/-} $n = 30$ mice, ICOS^{+/-} $n = 20$ mice. ***, $p < 0.0001$. The results shown are of one of the two representative experiments. **B**, Representative sections of pancreata from 14-wk-old ICOS^{-/-} or ICOS^{+/-} female NOD littermates were stained with H&E to determine severity of insulinitis and loss of islet cells. Female ICOS^{-/-} mice have minimal insular inflammation (*a*) or loss of islet cell (arrow, *inset b*) in contrast to the severe insular inflammation observed in ICOS^{+/-} mice (*c*) with marked reduction in islet cells (arrow, *inset d*). Scale bars = 500 (*a*, *c*) and 100 (*b*, *d*) μ m. **C**, Quantification of the severity of the insular inflammation (SI) loss of Islets (SL). Analysis of pancreata from ICOS^{-/-} and ICOS^{+/-} female NOD littermates. ICOS^{-/-} $n = 20$ mice, average age 25 wk ICOS^{+/-} $n = 20$ mice, Average age 17 wk. ***, $p < 10E-08$ (SI) and $p < 10E-07$ (SL).

and data not shown) We conclude that the presence of ICOS is necessary for the development of the clinical symptoms of diabetes in NOD mice.

Previous experiments have shown that blocking of both ICOS and CD40L (CD154) by mAbs led to a synergistic effect of prolonging islets graft survival and decreasing the incidence of diabetes in NOD mice (29). In contrast, monotherapy with anti-ICOS Abs resulted in only a limited protection from diabetes (29). Paradoxically, treatment with anti-ICOS Abs was also reported to

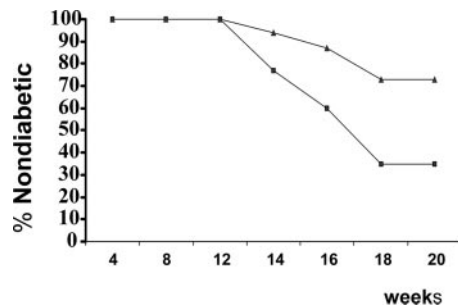


FIGURE 2. Protection from diabetes after anti-B7h treatment. NOD female mice were injected with (▲) or without (■) 100 μ g anti-B7h (anti-B7RP1) Ab at 3, 3.5, 4, 6, and 8 wk. Urine glucose levels were monitored weekly starting when mice reached the age of 10 wk. The increased glucose urine levels were verified by measuring blood glucose content exceeding 250 mg/dl. $n = 30$ mice. **, $p < 0.02$.

result in the impaired function of the intrapancreatic Tregs and increased insulinitis (19). Such opposing results of experiments that used anti-ICOS Abs may reflect either incomplete blocking of ICOS, complications caused by agonist vs antagonist effects on receptors, or different roles of ICOS during the early or late stages of the autoimmune process as was suggested previously for the role of ICOS in EAE (5, 20, 21). Given the conflicting results obtained with anti-ICOS Ab treatment, we decided to use an Ab against B7h (B7RP-1, ICOSL, GL-50), the only known partner for ICOS in mice to recapitulate our findings in NOD mice with a genetic deletion of ICOS (34). The treatment with anti-B7h Ab has been shown before to be more effective in ameliorating the symptoms of murine lupus nephritis than a therapy with anti-ICOS Ab (11). We treated NOD females with anti-B7h Ab (31) starting at 3 wk of age and found 73% remained nondiabetic in a treated group, as opposed to 35% nondiabetic in a control group (Fig. 2). We conclude that blocking of the ICOS-B7h axis results in a decreased incidence of diabetes, in agreement with our results obtained using NOD ICOS^{-/-} mice.

Hyperglycemia, a hallmark of diabetes, is caused by the progressive destruction of the β cells that begins in NOD mice several weeks before the onset of the clinical symptoms (22). We recovered pancreata from ICOS^{-/-} and ICOS^{+/-} littermates, performed microscopic analysis of the islets and scored the severity of inflammation and severity of islets loss (Fig. 1, B and C). We observed severe insulinitis and loss of islets in the ICOS^{+/-} NOD mice (Fig. 1B (c, d) and C). In contrast, we found that an overwhelming majority of the ICOS^{-/-} NOD female mice had normal numbers of islets and only a few animals developed marginal levels of inflammation of the islets (Fig. 1B (a, b) and C). We conclude that normoglycemia in ICOS^{-/-} NOD mice is maintained by the unaltered islets.

ICOS is important for the development of germinal centers in lymphoid tissues, isotype switching of immunoglobulins, and the production of normal serum immunoglobulins levels, particularly IgG1 and IgE as reviewed in Ref. 34. The role of autoantibodies in the development of diabetes remains controversial, nevertheless, the presence of Abs specific for insulin and GAD65 are an important diagnostic tool in assessing the course and severity of diabetes (35). We measured the levels of anti-GAD65 and anti-insulin immunoglobulins in sera from ICOS^{-/-} and ICOS^{+/-} littermates (Fig. 3) and we found a 3–5-fold reduction of autoantibodies levels in ICOS^{-/-} mice compared with ICOS^{+/-} littermates. We conclude that decreased production of autoantibodies correlates with the absence of diabetes in ICOS^{-/-} NOD mice. We further conclude that ICOS plays a different role in the development of dia-

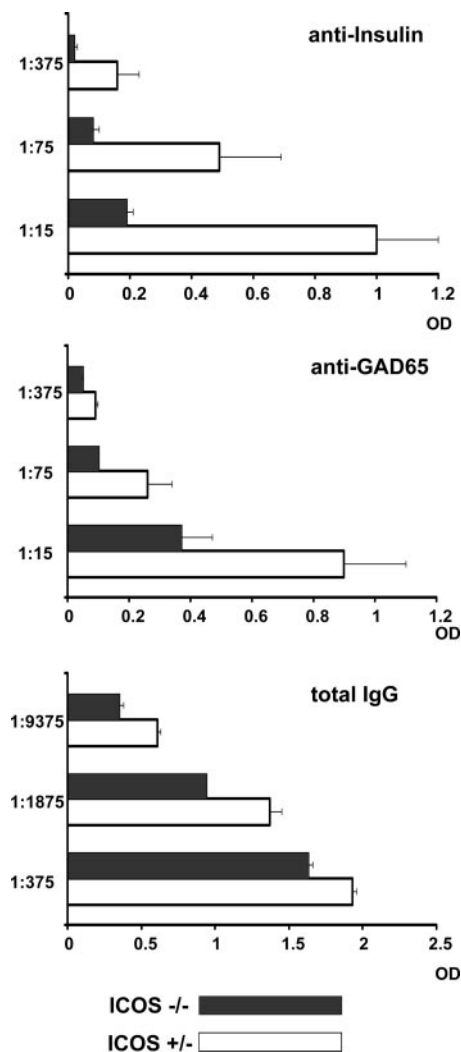


FIGURE 3. Diminished production of autoantibodies in the absence of ICOS. Sera from 13- to 15-wk-old ICOS^{-/-} and ICOS^{+/-} NOD female littermates were collected and analyzed independently by ELISA for the presence of anti-GAD65, anti-insulin Abs, and total IgG. The results of serial dilutions are average OD readings, error bars represent SEM. ICOS^{-/-}, $n = 6$ mice; ICOS^{+/-}, $n = 6$ mice; anti-insulin (1/15), **, $p < 0.02$; anti-GAD65 (1/15), *, $p < 0.05$; total IgG (1/375), ***, $p < 0.0002$; Closed bars ICOS^{-/-}, open bars ICOS^{+/-}.

betes than other members of the CD28/CTLA-4 family; specifically, CD28, CTLA-4, or PD-1, that when absent, all exacerbate the symptoms of T1 diabetes mellitus (23, 24, 26).

The role of ICOS in Tregs in NOD mice

Ag-experienced T cells are critically important for the development of diabetes in NOD mice and ICOS expression correlates with activation of T cells as reviewed in Refs. 22 and 34. To determine the pattern of ICOS expression in prediabetic animals, we examined by flow cytometry surface Ags on peripheral lymphocytes in NOD mice (Fig. 4 and data not shown). We found ICOS to be expressed predominantly by CD4 T cells. Approximately 15% of these lymphocytes (~5% of total lymphoid cells) were positive for ICOS in ICOS^{+/-} littermates; and as expected no ICOS expression was found on T cells from ICOS^{-/-} littermates (Fig. 4A). In agreement with previous reports, (2–9), ICOS^{-/-} NOD mice did not exhibit clear abnormalities in the development of a different population of lymphocytes (Fig. 4 and data not shown).

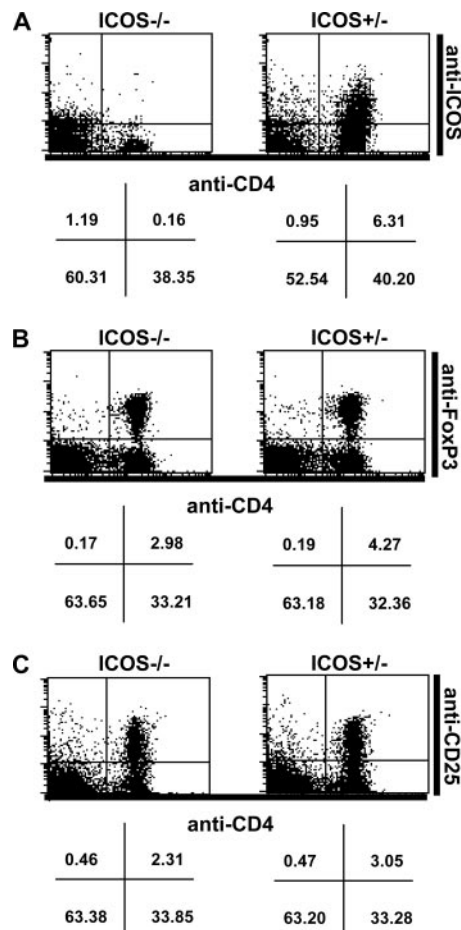


FIGURE 4. Surface and intracellular Ag expression in T cells without ICOS. Cells from lymph nodes and spleens of 6-wk-old female ICOS^{-/-} or ICOS^{+/-} NOD littermates were analyzed by flow cytometry. **A**, The plots show staining with anti-CD4 and anti-ICOS intensity gated on the live cells. The numbers represent the percentage of cells from each corresponding quadrant. **B** and **C**, The plots show staining with anti-CD4 and anti-FoxP3 (**B**) or anti-CD25 (**C**) intensity gated on the fixed/permeabilized or live cells. The numbers represent the percentage of cells from each corresponding quadrant. FoxP3 (percentage of FoxP3 positive; ***, $p < 0.00048$; $n = 6$ mice, percentage of CD25 positive; **, $p < 0.002$, $n = 6$ mice). The results shown are of one of the three representative experiments.

T cell responses can be influenced by the action of Tregs and ICOS has been implicated in establishing the homeostasis of anti-diabetogenic Tregs in the prediabetic lesions (19). To determine the impact of the absence of ICOS on Tregs in NOD mice, we compared the CD4/CD25 and CD4/FoxP3 double positive populations in peripheral T cells and T cells from the pancreatic lymph nodes of ICOS^{-/-} and ICOS^{+/-} littermates (Fig. 4, **B** and **C** and data not shown). We found ~30% reduction of the numbers of CD25 and FoxP3 positive CD4 T cells in the periphery and pancreatic draining lymph nodes of ICOS^{-/-} mice that nevertheless remained free of diabetes (see Fig. 1). Because the accumulation of lymphocytes into the pancreas is minimal in ICOS^{-/-} NOD mice, we could not directly investigate the role of ICOS in Tregs in the pancreas and consequently could not determine the function of ICOS in tissue-specific Tregs. We conclude that we do not find an evidence for the role of Tregs in the protection from diabetes we observe in ICOS^{-/-} NOD mice.

The impact of ICOS on effector responses by T cells in NOD mice

In mice, the ICOS gene is associated with Idd5 locus on chromosome 1. We chose to determine the allelic variation of the Idd5-specific D1Mit 74 and D1Mit 178/D1Mit 180 markers (*Materials and Methods*) in ICOS/NOD mice because these markers flank a genomic fragment that contains the ICOS locus but exclude the Idd5.2 locus. We confirmed by the available PCR protocols that both ICOS^{-/-} mice that remained completely diabetes-free and ICOS^{+/-} mice that developed diabetes (Fig. 1) harbored only NOD-specific alleles of D1Mit 74, D1Mit 178, and D1Mit 180 markers. NOD mice congenic for a non-NOD genomic fragment of the same or larger size than a region defined by D1Mit 74 and D1Mit 178/D1Mit 180 alleles have been shown to continue to develop diabetes at the rate of ~60–67% of the wild-type NOD mice (i.e., strains R1 (36) and R193 (37)). The partial protection from diabetes observed in such congenic strains has been proposed to be influenced by transcriptionally regulated expression of membrane-anchored (full length CTLA-4), ligand independent (liCTLA-4), and soluble (sCTLA-4) forms of CTLA-4 in NOD mice (32, 38). To examine directly the RNA expression levels of these different forms of CTLA-4 and also the expression of CD28 in CD4 T cells from ICOS^{-/-} or ICOS^{+/-} littermates, we performed real-time RT-PCR analysis of their transcripts (Fig. 5). We found that the different forms of CTLA-4 and the CD28 were expressed at similar levels in ICOS^{-/-} and ICOS^{+/-} mice. Therefore, we conclude, based on multiple independent lines of evidence, that presence of ICOS is required for a development of diabetes in NOD mice, although we cannot rule out a formal possibility that a gene or DNA element closely linked with ICOS might influence the observed phenotype of NOD ICOS mice.

ICOS expression has been showed to correlate with expression of CXCR5 on follicular B helper T cells. To determine whether the absence of ICOS had an impact on expression of CXCR5 by CD4 T cells in NOD mice we examined CXCR5 expression in CD4 T cells from ICOS^{-/-} and ICOS^{+/-} mice (Fig. 6). We found only a minimal 0.73-fold reduction in CXCR5 expression in T cells from ICOS^{-/-} mice. We conclude that absence of ICOS has a limited effect on expression of CXCR5 in peripheral T cells outside germinal centers consistent with previously published reports (39, 40).

Chemokine receptors such as CCR5 and CCR7 have been previously shown to modulate the function of either diabetogenic or Tregs (41–43). We examined the expression of such chemokine receptors in CD4 T cells from ICOS^{-/-} and ICOS^{+/-} mice and found that the absence of ICOS did not affect the expression levels of these chemokine receptors (Fig. 6).

Th1 T cells are thought to play a pathologic role in diabetes and costimulation through the ICOS of T cells activated under different conditions promotes production of Th1, Th2, or Th17 cytokines as reviewed in Ref. 34. In an attempt to define the type of T cell responses promoted by ICOS in NOD mice, we examined the cytokine production profile in total T cells ex vivo by flow cytometry (Fig. 7A). We found that ~2% of peripheral CD4 T cells from ICOS^{+/-} mice produced IFN- γ and the majority of IFN- γ producers coexpressed ICOS on their surface. In contrast, the frequency of IFN- γ positive T cells in ICOS^{-/-} littermates was decreased. We found only a minimal production of IL-4 or IL-17 by CD4 T cells from either ICOS^{-/-} or ICOS^{+/-} mice (data not shown). To examine directly the IFN- γ RNA expression levels in CD4 T cells, we performed real-time RT-PCR analysis of IFN- γ transcripts in freshly isolated T cells from either ICOS^{-/-} or ICOS^{+/-} mice (Fig. 7B). We found that in the absence of ICOS,

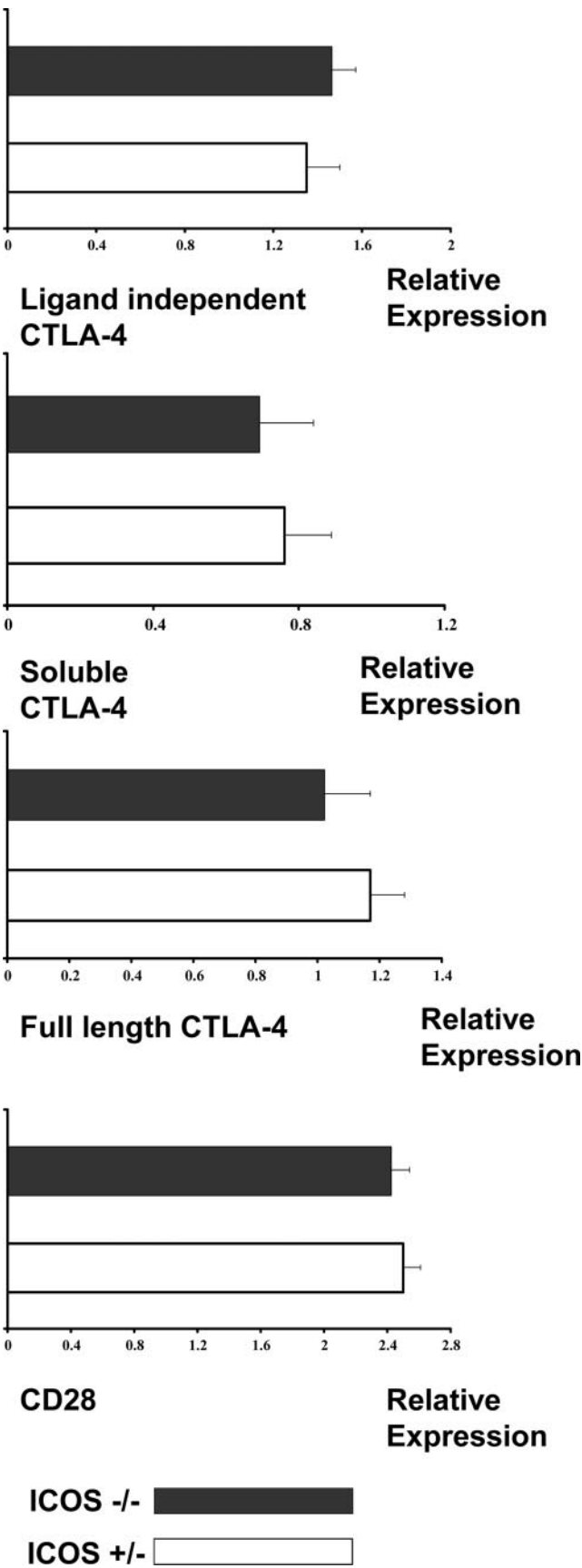


FIGURE 5. Relative expressions of CTLA-4 and CD28 in T cells without ICOS. Transcripts from CD4 cells from lymph nodes and spleens of 6- to 8-wk-old female ICOS^{-/-} or ICOS^{+/-} NOD littermates were analyzed by quantitative real-time RT-PCR. The results are normalized for expression of hypoxanthine phosphoribosyltransferase and are averages; error

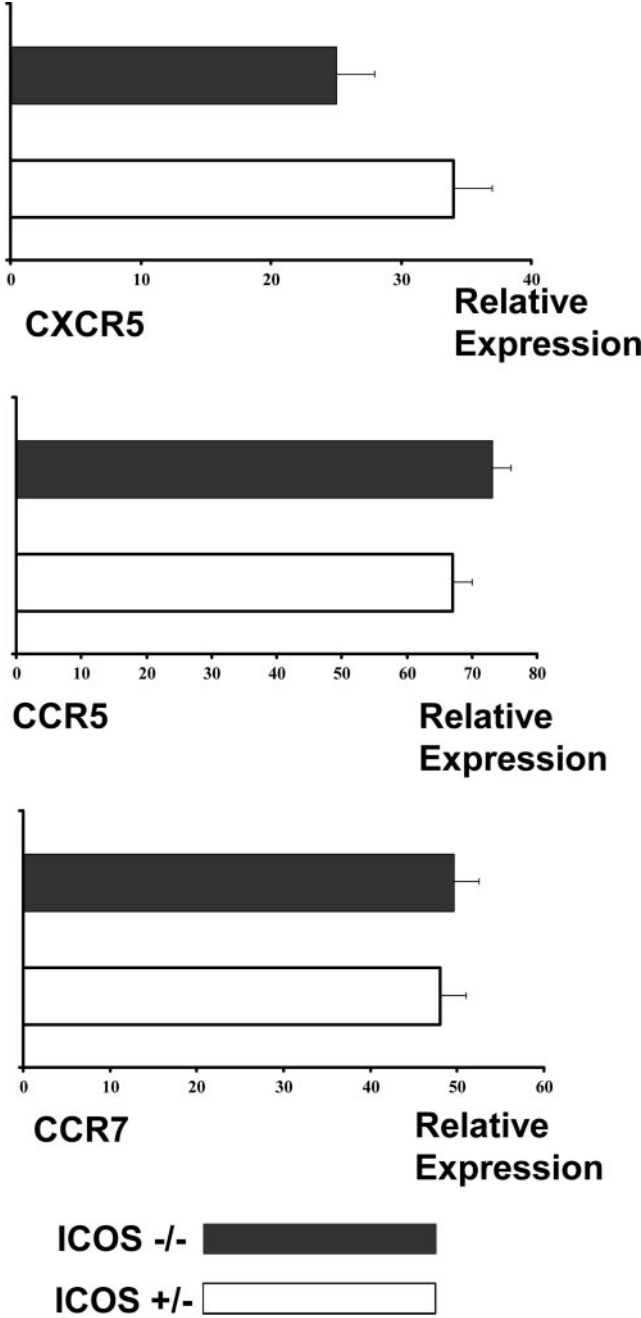


FIGURE 6. Relative expressions of chemokine receptors in T cells without ICOS. Transcripts from CD4 cells from lymph nodes and spleens of 6–8 wk old female ICOS^{-/-} or ICOS^{+/-} NOD littermates were analyzed by quantitative real-time RT-PCR. The results are normalized for expression of hypoxanthine phosphoribosyltransferase and are averages; error bars represent SEM. ICOS^{-/-}, *n* = 5 mice; ICOS^{+/-}, *n* = 5 mice; Closed bars, ICOS^{-/-}; open bars, ICOS^{+/-}. CXCR5, ***, *p* < 0.0001; pCCR5, ***, *p* > 0.7; CCR7, ***, *p* > 0.8.

IFN- γ RNA expression was decreased ~3-fold. We conclude that IFN- γ production by NOD T cells is associated with the expression of ICOS and in the absence of ICOS, the total expression of IFN- γ by T cells is decreased.

bars represent SEM. ICOS^{-/-}, *n* = 5 mice; ICOS^{+/-}, *n* = 5 mice; LiCTLA-4, ***, *p* > 0.56; sCTLA-4, **, *p* > 0.39; full length CTLA-4, ***, *p* > 0.73; CD28, ***, *p* > 0.51. Closed bars, ICOS^{-/-}; open bars, ICOS^{+/-}. The results shown are of one of the three representative experiments.

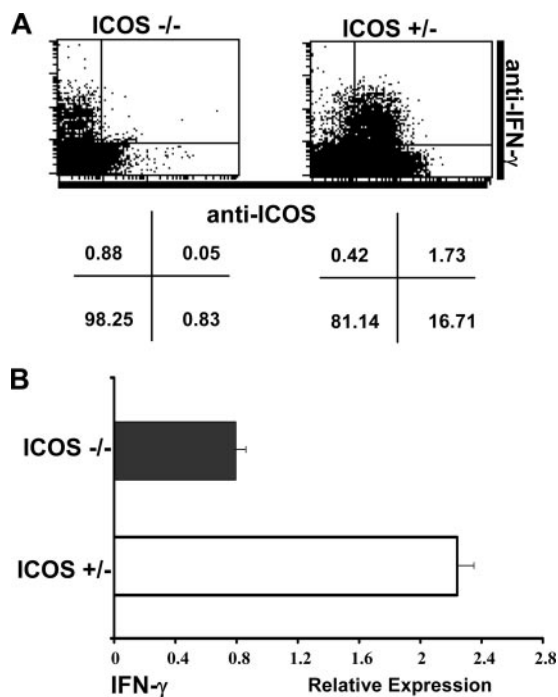


FIGURE 7. Diminished production of IFN- γ in absence of ICOS. **A**, The plots show staining with anti-ICOS and anti-IFN- γ intensity gated on the restimulated fixed/permeabilized CD4 positive cells from 6-wk-old female ICOS^{-/-} or ICOS^{+/-} NOD littermates. The numbers represent the percentage of cells from each corresponding quadrant. The results are from one of three similar experiments. **B**, IFN- γ transcripts from CD4 cells from lymph nodes and spleens of 6–8 wk old female ICOS^{-/-} or ICOS^{+/-} NOD littermates were analyzed by quantitative real-time RT-PCR. The results are normalized for expression of hypoxanthine phosphoribosyl-transferase and are averages, error bars represent SEM. ICOS^{-/-}, $n = 5$ mice; ICOS^{+/-}, $n = 5$ mice, ***, $p < 0.0001$.

Discussion

Our experiments show that ICOS is involved in producing the pathologic changes that underlie the development of diabetes mellitus in NOD mice and therefore the absence of functional ICOS might be therapeutic in this disease. Other members of the CD28/CTLA-4 family of costimulatory molecules such as CD28, CTLA-4, and PD-1 modulate the function of diabetogenic T cells to prevent islet inflammation and the ensuing hyperglycemia, but the role of ICOS in the development of insulinitis and diabetes remained unclear. We found ICOS^{-/-} mice to be spared from insulinitis, to have low titers of autoantibodies, and to remain normoglycemic throughout their lives (Figs. 1 and 3). Consistent with this, we could also block ICOS function by using anti-B7h Ab and we achieved an ~50% suppression of diabetes incidence in treated NOD female mice (Fig. 2). The blocking of ICOS function with Abs may be either incomplete or reflect its different roles during the early or late stages of the autoimmune process as was suggested previously. In EAE, blockade of ICOS immediately after the induction of the disease exacerbates the encephalomyelitis, a result consistent with data obtained from mice with a targeted deletion of ICOS; however, a delayed blockade of ICOS was found to ameliorate the symptoms of EAE (5, 20, 21). Additionally, injection of Abs such as anti-ICOS, can also impact on T cell function by a variety of different mechanisms including Fc receptor and complement mediated effects. Our results show that early absence of function of ICOS that is achieved in animals with a targeted deletion of ICOS renders a complete protection from diabetes. These

results argue for a development of a better method to disable ICOS function in vivo as a way of a therapeutic intervention during early stages of diabetes.

ICOS is expressed on Tregs in the prediabetic pancreatic lesions and the function of such Tregs has been found to be dependent on ICOS (19). Because the accumulation of lymphocytes in the pancreas is only minimal in ICOS^{-/-} NOD mice, we could not investigate the role of ICOS on Tregs in the pancreas. However, we observed an ~30% reduction in frequencies of FoxP3 positive CD4 T cells in the periphery and pancreatic draining lymph nodes of ICOS^{-/-} mice (Fig. 4). Because ICOS^{-/-} mice nevertheless remained free of diabetes, we were unable to find evidence that such a potentially impaired function of peripheral Tregs in the ICOS^{-/-} mice might contribute to the disease process. In some autoimmune models, such as EAE, ICOS has been shown to play both a protective and disease-inducing role (5, 20, 21). It therefore remains possible that, in addition to its role during the early induction of the autoimmunity resulting in lymphocytes infiltration in pancreas, ICOS may also be required for later, T-regulatory dependent mechanisms that operate inside pancreatic lesions to limit the pathological process, consistent with a previous report (19). Therefore, our results showing a 30% reduction of FoxP3 positive T cells in the absence of ICOS can be consistent with a role of ICOS in a homeostasis of the Tregs (19, 44). How different time- and tissue-specific functions of ICOS in various T cell types may be balanced remains unclear at this point and will be a subject of future investigation.

Our results establish a role of ICOS during the early processes leading to insulinitis and diabetes. The majority of ICOS^{-/-} mice have unaltered islets and the few mice that developed limited inflammation remained nevertheless protected from hyperglycemia and clinical diabetes (Fig. 1). This suggests that ICOS may be required both for initial induction and the early progression of islet inflammation that typically leads to destruction of islets and diabetes.

We did not find major differences in the expression of immunomodulatory molecules between T cells from ICOS^{-/-} and ICOS^{+/-} NOD mice (Figs. 4, 5, and 6). This is consistent with the lack of significant findings in the phenotype of ICOS deficient T cells in the periphery reported previously (2–9). The major reported difference between the T cells from young ICOS^{-/-} and ICOS^{+/-} mice appears to be the impairment of germinal center, CXCR5 positive CD4 T cells observed in absence of ICOS (39, 40). This leads to defects in isotype switching and consequently lower levels of serum immunoglobulins. Consistent with that, we find lower titers of insulin and GAD65 specific autoantibodies in NOD ICOS^{-/-} animals (Fig. 3).

The function of proinflammatory T cells that move from the periphery to infiltrate pancreatic islets is well-established in the pathogenesis of diabetes and the pathologic role of IFN- γ -producing cells in development of diabetes has been shown by numerous studies recently reviewed in Ref. 22. Absence of IFN- γ delays but does not completely prevent the onset of diabetes (22, 45, 46). We found that production of IFN- γ by CD4 T cells in NOD mice correlated with ICOS expression and that IFN- γ expression was decreased in the absence of ICOS (Fig. 7), but we did not find a change in other major cytokines such as IL-17 or IL-4 (data not shown). This is in agreement with a role of ICOS in stimulating the production of IFN- γ in human and mouse T cells under different pathologic conditions such as T cells activated with experimental Ags or T cell responses in patients to infection with *Mycobacterium tuberculosis* (Fig. 7 and Refs. 8, 21, 22). Our results are also consistent with the

reported role of ICOS in the development of some other forms of spontaneous autoimmunity such as murine lupus (11, 17). These studies found that the absence of ICOS ameliorated lymphadenopathy and vasculitis but had a limited or no effect on the severity of the glomerulonephritis (17). Such amelioration of symptoms of autoimmunity in the absence of ICOS was explained in these studies by decreased serum levels of autoantibodies and lower ratio of Th1/Th2 effector T cells (17). In contrast, diabetes is mostly a T cell-driven autoimmunity that starts early in life and the accumulating decrease in autoinsulin and anti-GAD65 autoantibodies is unlikely to have any role in the observed protection from diabetes in the absence of ICOS. However, a clear defect in a Th1 response observed in vivo in ICOS^{-/-} NOD mice (Fig. 7) is likely to have a profound inhibitory effect on the early development of insulinitis. Given the pleiotropic functions of ICOS, it also remains possible that in addition to its role in stimulating the Th1 cytokine response, ICOS might affect some other, still unidentified aspects of T cell biology that are relevant to the induction of diabetes. It is possible that fine-tuning of the balance between Th1 and Th2 responses in vivo by ICOS might be critical for the initiation and progression of insulinitis. We propose that ICOS plays a positive role in promoting a type 1 autoimmune response that ultimately leads to the destruction of islets and to diabetes. Our results suggest a potential therapeutic benefit of blocking ICOS in autoimmune diabetes.

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Disclosures

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