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Regulates ICOS Expression and Modulates Murine Experimental  
Autoimmune Encephalomyelitis <sup>1</sup> ✓**

Bernhard Greve; ... et. al

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# The Diabetes Susceptibility Locus *Idd5.1* on Mouse Chromosome 1 Regulates ICOS Expression and Modulates Murine Experimental Autoimmune Encephalomyelitis<sup>1</sup>

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Linkage analysis and congenic mapping in NOD mice have identified a susceptibility locus for type 1 diabetes, *Idd5.1* on mouse chromosome 1, which includes the *Ctla4* and *Icos* genes. Besides type 1 diabetes, numerous autoimmune diseases have been mapped to a syntenic region on human chromosome 2q33. In this study we determined how the costimulatory molecules encoded by these genes contribute to the immunopathogenesis of experimental autoimmune encephalomyelitis (EAE). When we compared levels of expression of costimulatory molecules on T cells, we found higher ICOS and lower full-length CTLA-4 expression on activated NOD T cells compared with C57BL/6 (B6) and C57BL/10 (B10) T cells. Using NOD.B10 *Idd5* congenic strains, we determined that a 2.1-Mb region controls the observed expression differences of ICOS. Although *Idd5.1* congenic mice are resistant to diabetes, we found them more susceptible to myelin oligodendrocyte glycoprotein 35–55-induced EAE compared with NOD mice. Our data demonstrate that higher ICOS expression correlates with more IL-10 production by NOD-derived T cells, and this may be responsible for the less severe EAE in NOD mice compared with *Idd5.1* congenic mice. Paradoxically, alleles at the *Idd5.1* locus have opposite effects on two autoimmune diseases, diabetes and EAE. This may reflect differential roles for costimulatory pathways in inducing autoimmune responses depending upon the origin (tissue) of the target Ag. *The Journal of Immunology*, 2004, 173: 157–163.

The centromeric end of mouse chromosome 1 includes a cluster of genes encoding the costimulatory molecules CD28, CTLA-4, and ICOS. Linkage analysis and congenic mapping in NOD mice have identified a susceptibility locus for type 1 diabetes, *Idd5.1*, which includes the *Ctla4* and *Icos* genes (1–5). Besides diabetes, numerous human autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, celiac disease, and autoimmune thyroiditis) have been mapped to a syntenic region on human chromosome 2q33 and are associated with single nucleotide polymorphisms (SNPs)<sup>4</sup> in the CTLA-4 gene (6–15).

The importance of the costimulatory molecules CD28, CTLA-4, and ICOS and their isoforms in regulating T cell activation, expansion, and effector functions makes these genes candidates for genetic regulation of autoimmune diseases. CD28 is constitutively expressed on T cells, and its ligation by its ligands (B7.1 and B7.2) provides essential positive signals for T cell activation, expansion, and survival. The lack of either CD28 or its ligands makes mice more resistant to experimental autoimmune encephalomyelitis (EAE) while enhancing diabetes (16–20). CTLA-4 surface expression is up-regulated after T cell activation and, in contrast to CD28, delivers a negative signal to the T cell. CTLA-4-deficient mice develop a severe lymphoproliferative disorder and multiorgan autoimmune disease (21, 22). Differences in mRNA levels of a recently described novel CTLA-4 splice variant (ligand-independent CTLA-4 (liCTLA-4)), which lacks the B7.1 and B7.2 binding domain have been found in T cells of diabetes-susceptible NOD mice and resistant C57BL/10 (B10) or NOD.B10 *Idd5.1* mice.<sup>5</sup> A synonymous SNP present in exon 2 of the mouse CTLA-4 gene has been proposed to be the molecular basis of *Idd5.1* by determining the efficiency of liCTLA-4 mRNA splicing (15). Another costimulatory molecule, ICOS, is up-regulated on T cells upon activation and provides a positive signal for T cell activation when cross-linked with its ligand B7h (23–25). As costimulation through ICOS promotes IL-10 and IL-4 production, it has been suggested that ICOS may play a critical role in Th2 differentiation (26). Blockade of ICOS costimulation during the induction phase enhanced EAE (27), and ICOS-deficient mice are more susceptible to EAE even on a genetically resistant background (28). These functional data demonstrate a critical role of the costimulatory molecules CD28, CTLA-4, and ICOS in regulating autoimmune diseases.

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<sup>4</sup> Abbreviations used in this paper: SNP, single nucleotide polymorphism; EAE, experimental autoimmune encephalomyelitis; flCTLA-4, full-length CTLA-4;

liCTLA-4, ligand-independent CTLA-4; ΔMFI, Δ mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein.

In the present study the expression of the costimulatory molecules CD28, full-length CTLA-4 (fICTLA-4), and ICOS on T cells of NOD, B6, B10, and *Idd5.1* congenic mice was analyzed. We found strain-specific differences in ICOS and, to a lesser extent, fICTLA-4 expression and determined that the *Idd5.1* region is responsible for the ICOS expression differences. To investigate whether *Idd5.1* is a shared susceptibility locus for diabetes and EAE, we tested *Idd5.1* congenic mice for myelin oligodendrocyte glycoprotein 35–55 (MOG<sub>35–55</sub>)-induced EAE. Unexpectedly, diabetes-resistant *Idd5.1* congenic mice developed more severe EAE compared with NOD mice. This difference correlated with higher expression of ICOS and greater production of IL-10 by activated NOD T cells compared with *Idd5.1*-derived T cells.

## Materials and Methods

### Mice

NOD and NOD.B10 *Idd5* R426, R46, and R974 mice were purchased from Taconic Farms (Germantown, NY). The R974 and R46 strains are available from Taconic Farms through the Emerging Models Program (lines 974 and 2193, respectively). B6 and B10 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions at the animal facility of Harvard Institutes of Medicine (Boston, MA).

### Expression analysis of costimulatory molecules by flow cytometry

Spleen cells from 6- to 10-wk-old female mice were stained in V-bottom, 96-well plates using the following FITC- or PE-labeled Abs: CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone 3C7), and CD28 (clone 37.51; all from BD PharMingen, San Diego, CA). For ICOS and OX-40 staining we used FITC-labeled or biotinylated Abs (clones 7E.17G9 and ACT35, respectively; BD PharMingen), followed by either FITC- or PE-labeled streptavidin. For measurement of CTLA-4 expression, cells were stained with anti-CD4 or -CD8, fixed and permeabilized (Cytofix/Cytoperm; BD PharMingen), and subsequently stained with a PE-labeled CTLA-4 Ab (UC10–4F10-11; BD PharMingen). Appropriate isotype controls were used in all experiments, and all experiments also included 7-amino-actinomycin D staining for the identification of dead or dying cells.

For measurements of induced expression, single-cell suspensions of spleen cells were prepared and activated in the presence of 1  $\mu\text{g}/\text{ml}$  of Con A in HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine and penicillin/streptomycin for different time periods. Four-color flow cytometry was performed on a FACSsort (BD Biosciences, Mountain View, CA), and data were analyzed using FlowJo 3.4 software (Tree Star, San Carlos, CA). The number of positive cells compared with isotype control-stained cells was calculated using the overtone subtraction method (included in FlowJo software). The  $\Delta$  mean fluorescence intensity ( $\Delta\text{MFI}$ ) was calculated as the mean fluorescence intensity of the stained sample minus that of the respective isotype control sample.

### In vivo activation of T cells and induction of EAE

For in vivo activation, mice were injected with 2  $\mu\text{g}$  of anti-CD3 (clone 145-2C11, low endotoxin/no azide; BD PharMingen) and 5  $\mu\text{g}$  of anti-CD28 (clone 37.51; BD PharMingen) in 200  $\mu\text{l}$  of PBS i.v. into the tail vein. Spleen cells were subjected to FACS staining, as described above, 12 or 48 h after injection.

EAE was induced by immunization of age-matched female mice with 200  $\mu\text{g}$  of MOG<sub>35–55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK) dissolved in PBS and mixed in equal parts with CFA supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). Mice were injected s.c. in both flanks. On days 0 and 2, each mouse was additionally given 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) i.v. into the tail vein. The severity of clinical disease was assessed as follows: score 0 = no paralysis, 1 = limp tail, 2 = limp tail and weak gait, 3 = hind limb paralysis, 4 = fore limb paralysis, and 5 = death or moribund animal. Mice were given water and food on the bottom of the cage when they were paralyzed.

### Histopathology

Brains and spinal cords were removed from the mice 7–10 wk after induction of EAE and fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections of these were stained with Luxol Fast Blue-H&E for

light microscopy. Histological disease was quantitated by counting the inflammatory foci in meninges and parenchyma as previously described (29).

### Activation of CD4<sup>+</sup> T cells and measurement of proliferation and cytokine production

Flat-bottom, 96-well plates were coated with different concentrations of anti-CD3 in 50  $\mu\text{l}$  of PBS/well for 2 h at 37°C. Plates were washed twice with sterile PBS, and wells were further coated with either 3  $\mu\text{g}$  of anti-ICOS Ab (26) or rat IgG control in 50  $\mu\text{l}$  of PBS/well. After 2-h incubation at 37°C, plates were again washed twice with sterile PBS.

CD4<sup>+</sup> cells were purified using CD4<sup>+</sup> T cell enrichment columns (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. After lysis of RBC, single-cell suspensions of two spleens per column were incubated for 15 min with the Ab mixture contained in the kit and loaded onto the column. After eluting, cells were counted, resuspended in HL-1 medium supplemented with L-glutamine and penicillin/streptomycin, and loaded onto the coated plates. Cell suspensions contained 92–94% CD4<sup>+</sup> T cells, as verified by FACS analysis (data not shown).

After 72 h, supernatants were removed, and wells were pulsed with 1  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine/well (NEN, Boston, MA). Cells were harvested 18 h later, and [<sup>3</sup>H]thymidine incorporation was measured using a beta scintillation counter (model LS5000; Beckman Coulter, Fullerton, CA).

Cytokines were measured in the supernatants using a sandwich ELISA as described previously (30). Briefly, plates were coated with the appropriate capture anti-cytokine Ab in bicarbonate buffer (pH 8) for 2 h at 37°C. After blocking with blocking solution (Kirkegaard & Perry, Gaithersburg, MD), samples and different standard dilutions were loaded onto the plates and incubated overnight at 4°C. After extensive washing, plates were incubated with the respective biotinylated secondary Ab and subsequently with streptavidin-peroxidase. The following mAb pairs were used: IL-4 (BVD4-1D11, capture; BVD6-24G2, detection), IL-10 (JES5-2A5, capture; SXC-1, detection), and IFN- $\gamma$  (R4-6A2, capture; XMG1.2, detection). SureBlue solution was used as a peroxidase substrate, and the reaction was stopped using stop solution (both from Kirkegaard & Perry). Absorption was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Analysis of the data was performed using Microplate Manager III software (Bio-Rad).

### Statistical analysis

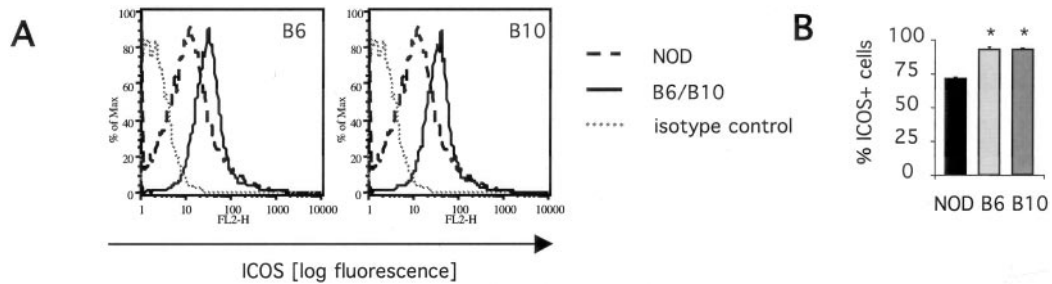
To compare ICOS and CTLA-4 expression in different mouse strains (NOD vs B6 and B10, NOD vs *Idd5.1* and R974), we used a two-way ANOVA model in which mouse strains and experiments are the two factors, and the log of the  $\Delta\text{MFI}$  measurements are the responses. Cumulative disease scores were calculated as the area under the curve for disease data of individual mice. The mean and SEM were calculated from pooled data. For between-group comparisons of clinical and histopathological data, Student's *t* test was used. To compare stimulated IL-10 secretion between NOD and *Idd5.1* mice, we used the nonparametric Wilcoxon test. Values of  $p < 0.05$  were considered statistically significant.

## Results

### ICOS is differentially expressed on unstimulated NOD and B6 or B10 T cells

ICOS is up-regulated on T cells after activation (28), but low levels of ICOS expression on a large proportion of unactivated T cells from NOD, B6, and B10 mice were detected using a biotinylated anti-ICOS mAb (Fig. 1). This finding might be due to the high sensitivity of using the combination of a biotinylated Ab with streptavidin-PE. There were, however, differences in the numbers of ICOS-positive unstimulated T cells among NOD and B6 or B10 mice. NOD mice had a lower percentage of ICOS-positive CD4, but not CD8, cells (Fig. 1B and data not shown).

To determine whether the expression differences are due to background genes or are controlled by the costimulatory locus *Idd5.1*, we measured ICOS expression in *Idd5.1* mice, which are congenic for a 1.2-cM B10-derived genomic interval encompassing the genes for CD28, CTLA-4, and ICOS on the NOD background (Fig. 2A). The same pattern of expression differences was detected, in that CD4<sup>+</sup> T cells from *Idd5.1* congenic mice showed higher numbers of ICOS<sup>+</sup> T cells compared with CD4<sup>+</sup> T cells from NOD mice (Fig. 2, B and C). Thus, expression differences in ICOS on unactivated CD4<sup>+</sup> T cells appear to be controlled by an



**FIGURE 1.** ICOS expression on splenic T cells from naive NOD and B6/B10 mice. Staining with biotinylated anti-ICOS Ab and streptavidin-PE as secondary reagent. *A*, Histogram overlay of B6 and B10 mice vs NOD mice gated on CD4<sup>+</sup>7-amino-actinomycin D<sup>-</sup> cells. *B*, Percentage of ICOS-expressing cells among CD4<sup>+</sup> T cells from unactivated B6, B10, and NOD mice. The means of four different mice in each group ± SEM are shown. \*, *p* < 0.0001 for NOD vs B6 or B10 (by two-way ANOVA).

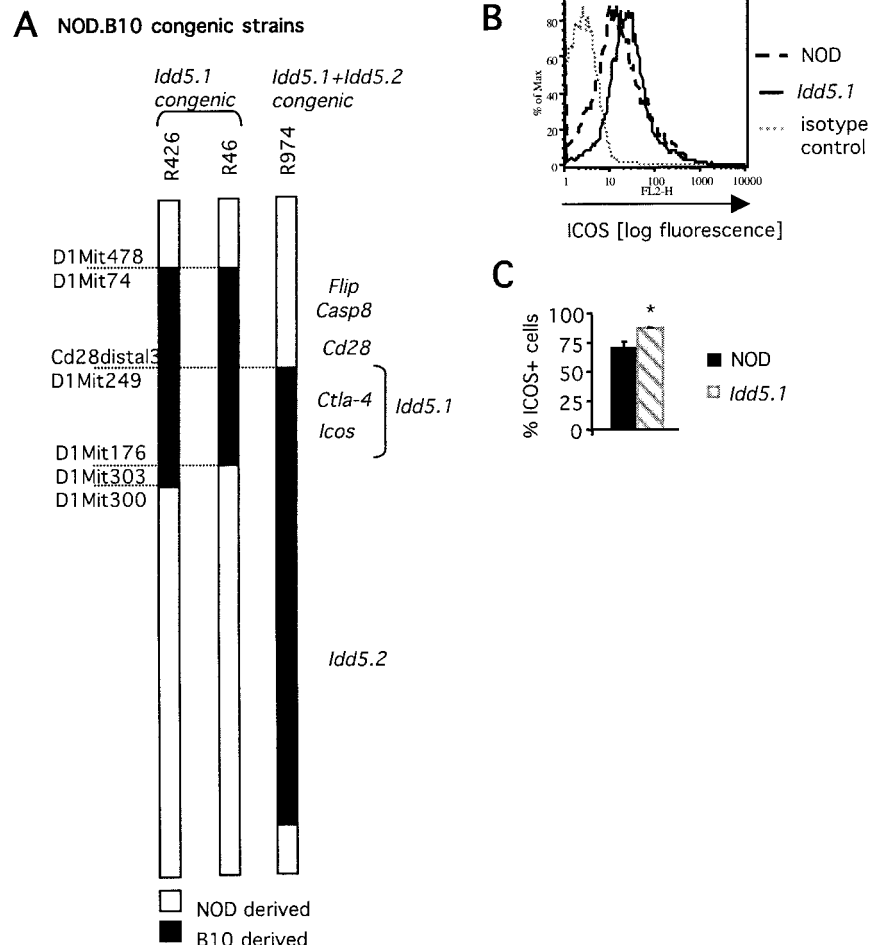
allelic variation at the costimulatory locus. As CD28 and CTLA4 are both included in the congenic interval of *Idd5.1* mice, we also assessed CD28 and CTLA4 expression in unactivated T cells. There were no differences in the level of CD28 expression between these two strains, and CTLA-4 could not be detected on unactivated T cells (data not shown).

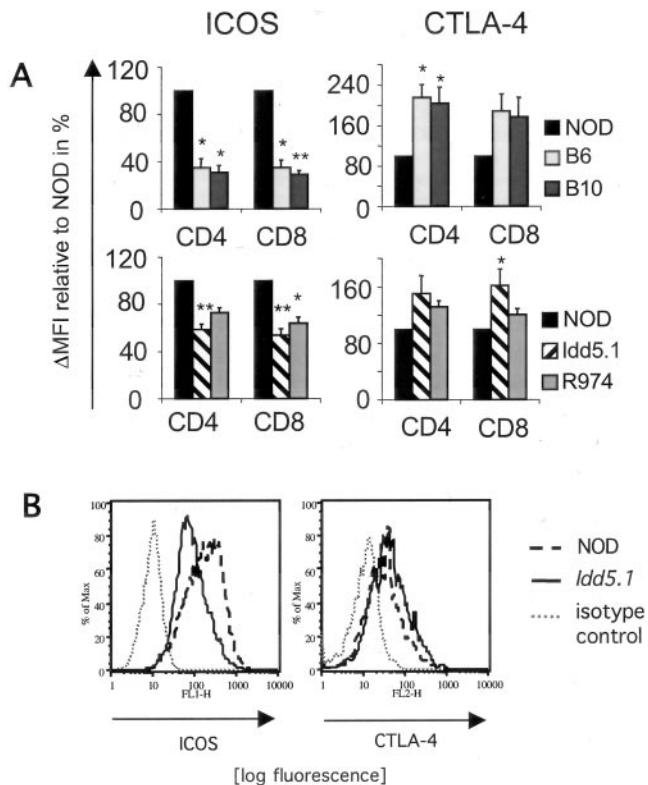
*ICOS and CTLA4 expression on activated T cells*

We next determined whether allelic differences at the *Idd5.1* locus also lead to differential expression of ICOS and CTLA-4 after activation of T cells. In these experiments we additionally tested another congenic mouse strain, R974, which recombines between *Cd28* (NOD derived) and *Ctla4* (B10 derived; Fig. 2*A*). The telo-

meric portion of the congenic interval in R974 mice also includes the *Idd5.2* locus. After in vitro Con A activation, ICOS and CTLA-4 expressions were maximal 48 h after stimulation (data not shown). As reported previously (26), we found that activation of T cells led to an increase in ICOS expression in all strains tested; however, the expression of ICOS on activated T cells from B6, B10, and *Idd5.1* congenic mice was significantly lower than that on NOD T cells (Fig. 3). In contrast, CTLA-4 expression was significantly higher in CD4<sup>+</sup> T cells from B6 and B10 mouse strains than in NOD T cells (Fig. 3). However, CTLA-4 expression was less consistent among individual mice than ICOS expression. The congenic R974 mice followed a pattern similar to that in *Idd5.1* congenic mice, in that their activated T cells showed lower

**FIGURE 2.** ICOS expression on splenic T cells from naive NOD and *Idd5.1* congenic mice. *A*, Map of the NOD.B10 congenic mouse strains used. R426 and R46 encompass ~1.2 cM of B10-derived genetic material. These two strains were used interchangeably in the experiments and are both referred to as *Idd5.1* congenics. Strain R974 recombines between *Cd28* (NOD derived) and *Ctla4* (B10 derived). The telomeric end of R974 additionally contains the type 1 diabetes locus *Idd5.2*. Shown on the left are microsatellite markers polymorphic in NOD and B10 mice that mark the boundaries of the *Idd5.1* congenic interval. The genes of interest in the congenic interval are shown on the right. *B*, Representative histogram showing ICOS expression in CD4<sup>+</sup> T cells from NOD and *Idd5.1* congenic mice. Data are representative of five mice per group. *C*, Percentages of splenic CD4<sup>+</sup> T cells in unstimulated NOD and *Idd5.1* mice (R46) that are ICOS positive. The values are the mean of five mice ± SEM. \*, *p* = 0.0001 (by two-way ANOVA).





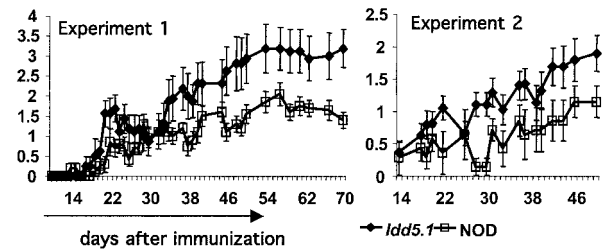
**FIGURE 3.** ICOS and CTLA-4 expression on activated T cells. *A*, Expression of ICOS and CTLA-4 on splenic T cells from NOD, B6, B10, *Idd5.1* (R46 and R426), and *Idd5.1+Idd5.2* (R974) mice. Spleen cells were stimulated with 1  $\mu\text{g}/\text{ml}$  Con A and stained for ICOS and CTLA-4 after 48 h.  $\text{CD4}^+$  ( $\text{CD8}^+$ ) or  $\text{CD4}^{\text{high}}$  ( $\text{CD8}^{\text{high}}$ ) cells were gated for analysis. The values are expression as  $\Delta\text{MFI}$  relative to NOD (percentage)  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$  (vs NOD, by two-way ANOVA). *B*, Histograms (representative of five or six experiments) of expression of inducible costimulatory molecules ICOS and CTLA-4 on  $\text{CD4}^+$  T cells from NOD and *Idd5.1* (R46 and R426) mice after in vitro stimulation for 48 h.

ICOS and higher CTLA-4 expression (Fig. 3A). Thus, the  $\sim 2.1$ -Mb overlapping interval between the strains that encompasses the genes for ICOS and CTLA-4, but not CD28, differentially regulates the expression of ICOS and, to a lesser extent, CTLA-4 on activated T cells. Furthermore, no differences in expression levels of OX40, another CD28-dependent costimulatory molecule, on activated T cells of NOD and *Idd5.1* congenic mice were found (data not shown).

We measured ICOS expression on spleen cells after in vivo administration of anti-CD3 plus anti-CD28 antibodies. Consistent with in vitro data, after 48 h NOD  $\text{CD4}^+\text{CD25}^+$  T cells expressed relatively higher amounts of ICOS compared with *Idd5.1* congenic-derived T cells (data not shown). Taken together, the in vitro and in vivo data suggest that ICOS expression on unstimulated and recently activated T cells in *Idd5.1* mice is greater than that in NOD mice. However, at the later stages of activation, T cells from NOD mice show a much higher ICOS expression compared with T cells from *Idd5.1* mice.

#### *Idd5.1* congenic mice develop more severe EAE than NOD mice

We next determined whether the differences in the expression of costimulatory molecules between NOD and *Idd5.1* congenic mice influence EAE. We performed two EAE experiments that included a total of 17 NOD and 27 *Idd5.1* congenic mice. In both experiments the *Idd5.1* congenic mice showed more severe EAE (Fig. 4) than the NOD mice. Although disease incidence was 100% in both



**FIGURE 4.** EAE in NOD and *Idd5.1* congenic mice. Disease course after immunization with 200  $\mu\text{g}$  of  $\text{MOG}_{35-55}$  in CFA and two i.v. injections with 100 ng of pertussis toxin. Shown are two experiments, including a total of 17 NOD and 27 *Idd5.1* congenic (R426) mice.

groups, *Idd5.1* congenic mice showed a significantly higher disease severity and cumulative disease scores than NOD mice (Table I). These clinical data are further supported by the histopathological data obtained from the brains and spinal cords of the animals, which revealed significantly higher numbers of inflammatory lesions in meninges and CNS parenchyma of *Idd5.1* mice compared with NOD mice (Table I).

#### Proliferation and cytokine production after costimulation through ICOS

ICOS has been found to be mainly expressed on Th2 cells, and costimulation through ICOS has been implicated in the regulation of EAE (27, 28). As our data showed that *Idd5.1* congenic mice develop more severe EAE than NOD mice, it is possible that the differential kinetics of ICOS expression between *Idd5.1* and NOD mice might influence proliferation and cytokine production after costimulation through ICOS. To test this hypothesis we analyzed  $\text{CD4}^+$  T cells from NOD and *Idd5.1* congenic mice after activation with anti-CD3 and anti-ICOS Abs. Purified  $\text{CD4}^+$  T cells were stimulated with different concentrations of anti-CD3, anti-ICOS, or rat IgG control Ab; proliferation was measured by [ $^3\text{H}$ ]thymidine incorporation assay; and supernatants were subjected to cytokine analysis by ELISA (Fig. 5).

Although ICOS costimulation in vitro had only modest effects on proliferation and  $\text{IFN-}\gamma$  secretion (Fig. 5, *A* and *B*), NOD mice produced significantly higher amounts of IL-10 than the *Idd5.1* congenic mice upon cross-linking with anti-ICOS Ab (Fig. 5*D*). IL-4 production was slightly enhanced in NOD-derived T cells compared with that in *Idd5.1* mice (Fig. 5*C*). These data suggest that differences in ICOS expression on the surface of  $\text{CD4}^+$  cells in NOD and *Idd5.1* mice result in marked changes in Th2 cytokine production after costimulation.

## Discussion

In the present study we address the regulation of expression of the costimulatory molecules CD28, CTLA-4, and ICOS by different alleles of the diabetes susceptibility locus *Idd5.1* on mouse chromosome 1. We show that 1) ICOS and CTLA-4 expression is regulated differently among diabetes-susceptible NOD and diabetes-resistant B6 or B10 mice; upon activation, diabetes-susceptible NOD mice express higher levels of ICOS than the resistant strains; 2) expression differences in ICOS among NOD and B6 or B10 mice are mainly due to a genomic segment of 2.1 Mb encompassing *Icos* and *Ctla4*, but excluding *Cd28*; 3) T cells of NOD mice respond to costimulation through ICOS with higher IL-10 production compared with *Idd5.1* mice; and 4) in contrast to diabetes, which is partially inhibited in *Idd5.1* mice,  $\text{MOG}_{35-55}$ -induced EAE is enhanced in *Idd5.1* congenic mice.

We demonstrate that T cells from diabetes-susceptible (NOD) and -resistant strains (B6 and B10) differentially up-regulate ICOS

Table I. Clinical disease and histopathological evaluation of MOG<sub>35-55</sub>-induced EAE in NOD and *Idd5.1* (R426) mice<sup>a</sup>

		Clinical Disease		Histopathology (number of lesions)		
		Maximal disease score	Cumulative disease score	Parenchyma	Meninges	Total
Expt. 1	NOD (n = 10)	2.8 ± 0.2	66.3 ± 4.6	12.1 ± 3.2	14.5 ± 4.0	26.6 ± 6.6
	<i>Idd5.1</i> (n = 8)	3.8 ± 0.4**	117.6 ± 22.7*	43.6 ± 15.4	39.5 ± 14.1	83.1 ± 29.3
Expt. 2	NOD (n = 7)	1.9 ± 0.2	22.4 ± 5.9	9 ± 1.8	7.6 ± 1.6	16.6 ± 3.0
	<i>Idd5.1</i> (n = 19)	2.7 ± 0.2***	42.3 ± 4.8**	16.2 ± 4.9	17 ± 5.2	33.1 ± 1
Total <sup>b</sup>	NOD (n = 17)	2.2 ± 0.1	28.0 ± 3.1	10.8 ± 1.9	11.6 ± 2.5	22.5 ± 4.0
	<i>Idd5.1</i> (n = 27)	2.9 ± 0.2***	46.4 ± 5.2****	24.3 ± 5.9**	23.7 ± 5.7	47.9 ± 11.5**

<sup>a</sup> Numbers are mean ± SEM.

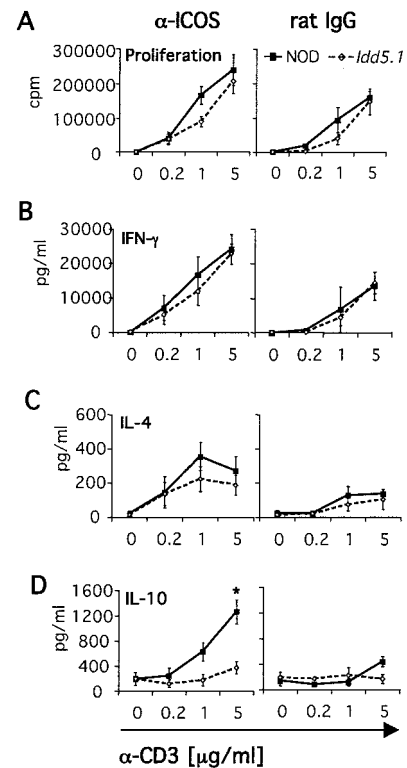
<sup>b</sup> For the calculation of total maximal disease scores and cumulative disease scores, data until day 50 were included from both experiments.  
\*,  $p < 0.07$ ; \*\*,  $p = 0.05$ ; \*\*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.005$ , NOD compared to *Idd5.1*.

and flCTLA-4, and that the differences in ICOS expression are genetically controlled by the newly defined, 2.1-Mb *Idd5.1* interval. Other studies investigating flCTLA-4 expression in NOD, B6, and NOD.B6 *Idd5.1* congenic mice showed similar results, with one study showing no expression differences of CTLA-4 in NOD and NOD.B6 *Idd5.1* after stimulation (4), and another suggesting a defect in CTLA-4 up-regulation on stimulated T cells in NOD mice compared with B6 mice (31). Our data suggest that there is relatively lower expression of CTLA-4 in the NOD-derived T cells compared with B6 or B10 T cells, but differences in ICOS expression are more consistent than those in CTLA-4 expression.

As CTLA-4 and ICOS are both dependent on CD28 costimulation, we investigated the possible effects of costimulation through CD28 on ICOS and CTLA-4. However, we found similar levels of CD28 on NOD and *Idd5.1* congenic T cells. In addition, another CD28-dependent costimulatory molecule, OX40, which is encoded on mouse chromosome 4, was not differentially expressed after Con A stimulation of NOD and *Idd5.1* T cells (data not shown). Furthermore, the expression of ICOS and CTLA-4 in the R974 strain, which carries B10-derived alleles of *Icos* and *Ctla4*, but an NOD-derived allele of *Cd28*, was similar to that found in *Idd5.1* congenic mice. Therefore, we have strong evidence that expression differences in ICOS (and flCTLA-4) are probably not due to differences in CD28 signaling.

Although the functional significance of ICOS expression on native T cells remains unclear, we further demonstrated that NOD CD4<sup>+</sup> cells, which express more ICOS on the cell surface after activation, respond better to costimulation through the ICOS costimulatory molecule. Cross-linking of ICOS with its ligand B7h has been shown to enhance the proliferation and production of various cytokines as well as provide help for Ig production by B cells (26, 32). ICOS has been shown to be particularly important in enhancing IL-10 production and development of Th2 cells (26, 33). Accordingly, we found only modest effects of ICOS costimulation on proliferation and IFN- $\gamma$  production. However, IL-10 production by NOD CD4<sup>+</sup> T cells was greatly enhanced after cross-linking of ICOS compared with *Idd5.1* congenic CD4<sup>+</sup> T cells. As activated *Idd5.1* T cells express significant amounts of ICOS on their cell surface, although to a lesser extent compared with NOD T cells, it may be that only a subset of high ICOS-expressing cells is responsible for the differences in IL-10 production between the strains. In fact, it has been shown recently that T cells, which

express high levels of ICOS, produce large amounts of IL-10 upon activation. In contrast, T cells expressing low or medium levels of ICOS produced IFN- $\gamma$ , IL-2, IL-3, and IL-6 or IL4, IL-5, and



**FIGURE 5.** Proliferation and cytokine production in CD4<sup>+</sup> T cells from NOD and *Idd5.1* (R46) mice. CD4<sup>+</sup> cells were purified using specific enrichment columns and stimulated in 96-well plates coated with the indicated amount of anti-CD3 (x-axis) plus 3 µg/ml anti-ICOS or rat IgG control Ab. A, Proliferation, measured as [<sup>3</sup>H]thymidine incorporation (cpm). IFN- $\gamma$  (B), IL-4 (C), and IL-10 (D) levels were measured by ELISA in supernatants after 72 h of stimulation. All concentrations are given as picograms per milliliter. The values are the mean of pooled data from three or four individual mice, with the SEM indicated. \*,  $p = 0.03$  (by Wilcoxon test).

IL-13, respectively (34). It has also been shown that ICOS expression on T cells after activation is higher in BALB/c mice compared with B6 mice. Importantly, this has been linked to higher Th2 cytokine production in T cell blasts from highly EAE-resistant BALB/c mice after costimulation through ICOS (35). Furthermore, it has been demonstrated that ICOS expression and IL-4 production correlated in various mouse strains (36). Thus, our results are in accord with previous findings in different mouse strains, but, in addition, demonstrate that differences in ICOS expression are controlled by the genomic interval encompassing the costimulatory locus.

EAE as well as autoimmune diabetes are polygenic diseases, and a number of gene regions have been implicated for harboring disease susceptibility genes (29, 37–40). Many of the gene regions conferring susceptibility to murine autoimmune models as well as to human autoimmune disorders overlap (41, 42). These observations have led to the hypothesis of shared autoimmune genes, but might also be due to clustering of relevant immune genes in the same genomic regions (41). For example *Bphs*, a locus controlling pertussis toxin-induced vasoactive amine sensitization elicited by histamine, is a common susceptibility locus for EAE and experimental autoimmune orchitis and has been shown to be identical with the histamine receptor H<sub>1</sub> (43, 44). Also, we have demonstrated that autoimmune diabetes and EAE share a susceptibility locus on chromosome 3 (*Idd3*), and we suggested that the NOD allele of IL-2 might predispose to both autoimmune diseases (45).

We now report enhanced MOG-induced EAE in *Idd5.1* mice compared with NOD mice. Thus, *Idd5.1* seems to differentially regulate autoimmune diabetes and EAE in NOD mice. Furthermore, the difference in susceptibility of NOD and *Idd5.1* congenic mice to EAE most likely relates to the differential expression of the costimulatory molecule ICOS.

The involvement of ICOS in EAE has previously been investigated. ICOS-deficient mice show exacerbated disease compared with wild-type mice (28). Accordingly, blockade of ICOS with Abs during the induction phase of EAE exacerbates disease (27). In this study we show that lower ICOS expression in activated T cells derived from *Idd5.1* mice correlates with lower IL-10 production in CD4<sup>+</sup> T cells after ICOS costimulation. The inhibitory role of IL-10 in the development of EAE has been demonstrated previously, in that IL-10 deficient mice develop more severe disease compared with wild-type mice, and overexpression of IL-10 protects from EAE (46). Therefore, our present data suggest that higher ICOS expression and the general ability of NOD mice to produce higher amounts of IL-10 upon costimulation with ICOS might lead to an ameliorated course of EAE.

The EAE disease phenotype contrasts with those of type 1 diabetes, because *Idd5.1* congenic mice are partially protected from diabetes (3, 5). The influence of IL-10 on diabetes in NOD mice, however, is highly dependent on both the time point and the tissue compartment in which it is released during the disease. Systemic administration of IL-10 or IL-10 fusion protein, particularly at later time points, leads to a reduction of disease (47, 48), whereas pancreatic overexpression of IL-10 leads to accelerated type 1 diabetes and is even sufficient to induce disease in resistant B10.H2<sup>g7</sup> congenic mice (49, 50). In this system, an ICAM-1-dependent pathway has been implicated in the acceleration of type 1 diabetes (51).

In view of the different effects of IL-10 in the two disease models, EAE and diabetes, the dynamic alterations of ICOS expression in NOD and *Idd5.1* congenic mice could lead to exacerbation of EAE and amelioration of diabetes in the same *Idd5.1* congenic mouse strain, depending on the time course and sites of expression and production of IL-10. Furthermore, as ICOS and CTLA-4 are reversibly regulated by the *Idd5.1* region, the balance of costimu-

latory signals may also be a factor that modulates each autoimmune disease differently. Local expression of B7-1/B7-2 and B7h might also vary both at priming sites and in the target organs, i.e., the CNS and the pancreas, thereby contributing to differences in susceptibility to the two autoimmune diseases. Therefore, different costimulatory pathways leading to the same cytokine secretion could result in different outcomes depending on the type of autoimmune disease.

The differential expression of ICOS in NOD and *Idd5.1* mice might be controlled directly by polymorphisms in the *Icos* gene or could be controlled by other molecules that are, in turn, genetically regulated by the *Idd5.1* locus. In the accompanying paper, Wicker and colleagues (5) show strain-specific differences in mRNA levels of the novel splice variant liCTLA-4, which are controlled by the *Idd5.1* locus. Although it is still unclear whether ICOS and liCTLA-4 regulate each other, it is possible that the expression of ICOS is controlled by variation in liCTLA-4 levels in T cells of the different mouse strains. Our data suggest that the autoimmune disease-associated variant liCTLA-4 inhibits T cell activation and suppresses cytokine production.<sup>5</sup> Thus, it is possible that a lower ICOS expression in NOD *Idd5.1* congenic mice is an indirect effect of the high expression of liCTLA-4 in this strain. To resolve this question we are currently backcrossing *Idd5.1* alleles of other mouse strains onto the NOD background. Investigating congenic mouse strains that have different combinations of haplotypes of the *Ctla4* and *Icos* genes will help to define further the genetic or molecular basis controlling the ICOS expression differences and the development of different organ-specific autoimmune diseases.

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