Original Article

Defective Induction of CTLA-4 in the NOD Mouse Is Controlled by the NOD Allele of *Idd3/IL-2* and a Novel Locus (*Ctex*) Telomeric on Chromosome 1

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Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4), or CD152, is a negative regulator of T-cell activation and has been shown to be associated with autoimmune diseases. Previous work has demonstrated a defect in the expression of this molecule in nonobese diabetic (NOD) mice upon anti-CD3 stimulation in vitro. Using a genetic approach we here demonstrate that a novel locus (Ctex) telomeric on chromosome 1 together with the *Idd3* (*Il-2*) gene confers optimal CTLA-4 expression upon CD3 activation of T-cells. Based on these data, we provide a model for how gene interaction between *Idd3* (*IL-2*), *Ctex*, and *Idd5.1* (*Ctla-4*) could confer susceptibility to autoimmune diabetes in the NOD mouse. Additionally, we showed that the *Ctex* and the *Idd3* regions do not influence inducible T-cell costimulator (ICOS) protein expression in NOD mice. Instead, as previously shown, higher ICOS levels in NOD mice appear to be controlled by gene(s) in the *Idd5.1* region, possibly a polymorphism in the *Icos* gene itself. *Diabetes* 55:538–544, 2006

T-cell stimulation is mediated by signaling through the antigen-receptor complex (T-cell receptor [TCR]/CD3) and through costimulatory molecules, providing either positive or negative signals important in the regulation of T-cell activation (1–3). Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) is one of the most well-characterized negative regulatory molecules (4–6). Direct evidence of a critical regulatory role for CTLA-4 comes from CTLA-4–deficient mice, which develop a severe lymphoproliferative disorder with multiorgan lymphocyte infiltration and die around 3–4 weeks of age (7,8). Further, growing evidence has been presented suggesting a role of the CTLA-4 molecule in autoimmune diseases both in humans and in animal models (9–16).

The nonobese diabetic (NOD) mouse is one of the most commonly used animal models for type 1 diabetes (17–20). It spontaneously develops insulitis that ends with the destruction of β-cells and overt diabetes. The mechanisms that control initiation and progression of this autoimmune process are unclear, but a role for CD28/CTLA-4 costimulatory pathways has been suggested in the early stages of the disease development (21,22). Further, genetic analysis of the NOD mouse model for type 1 diabetes has identified the *Ctla-4* gene as a major candidate for the *Idd5.1* diabetes susceptibility locus (10). More recently, Wicker et al. (15) confirmed that the susceptibility of diabetes conferred by the *Idd5.1* locus is associated with a splice form encoding a molecule lacking the CD80/CD86 ligand-binding domain, referred to as the ligand-independent isoform (liCTLA-4).

In accordance with the notion that CTLA-4 may play an important role in disease development of the NOD mouse, we previously reported that NOD T-cells express lower levels of CTLA-4 upon anti-CD3 stimulation in vitro compared with other mouse strains (23). Here, we have applied a congenic mapping approach to identify the genetic factor(s) underlying this discrepancy in CTLA-4 expression in NOD mice. We demonstrate that a novel locus (*Ctex*) in the distal part of the chromosome 1 together with the *Idd3* locus on chromosome 3 constitute the major factors mediating the observed difference in CTLA-4 expression levels. Moreover, we show that the defective expression of CTLA-4 in NOD T-cells activated through the CD3 complex in vitro can in part be overcome by the addition of exogenous interleukin (IL)-2. Together, these observations functionally link the two prime candidate genes for *Idd3* and *Idd5.1*, *Il-2*, and *Ctla-4*, respectively, and the novel *Ctex* locus, suggesting that their interaction contributes to the pathogenesis of diabetes in the NOD mouse.

RESEARCH DESIGN AND METHODS

C57BL/6 (B6) and NOD mice were originally obtained from Bomholtgaard (Ry, Denmark), and NOD.*Idd3* (NOD.B6[PL]-[D3Mit167-D3Mit94]) mice were obtained from The Jackson Laboratory (http://jaxmice.jx.org). NOD.R444 (14) were purchased from Taconic, Denmark. NOD.C1 mice were originated from a F2(B6×NOD) mouse that was backcrossed five times to a NOD mouse strain and intercrossed four to six times (see RESULTS). Sex- and age-matched animals were used at 3–10 weeks of age as indicated. In our colony, spontaneous diabetes occured in female NOD mice at an incidence of ~80% at 25 weeks of age. All animals were maintained at the animal facilities of Umeå University.

Cell suspensions were prepared from spleens of 3–10 week old mice in Hank's balanced salt solution. Erythrocytes were depleted with Gey's lysis buffer. Cells were cultured at 1×10^6/ml in RPMI-1640 medium containing 10%

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APC, allophycocyanin; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; FITC, fluorescein isothiocyanate; ICOS, inducible T-cell costimulator; IL, interleukin; LOD, logarithm of odds; mAb, monoclonal antibody; PE, phycoerythrin; TCR, T-cell receptor; QTL, quantitative trait locus.

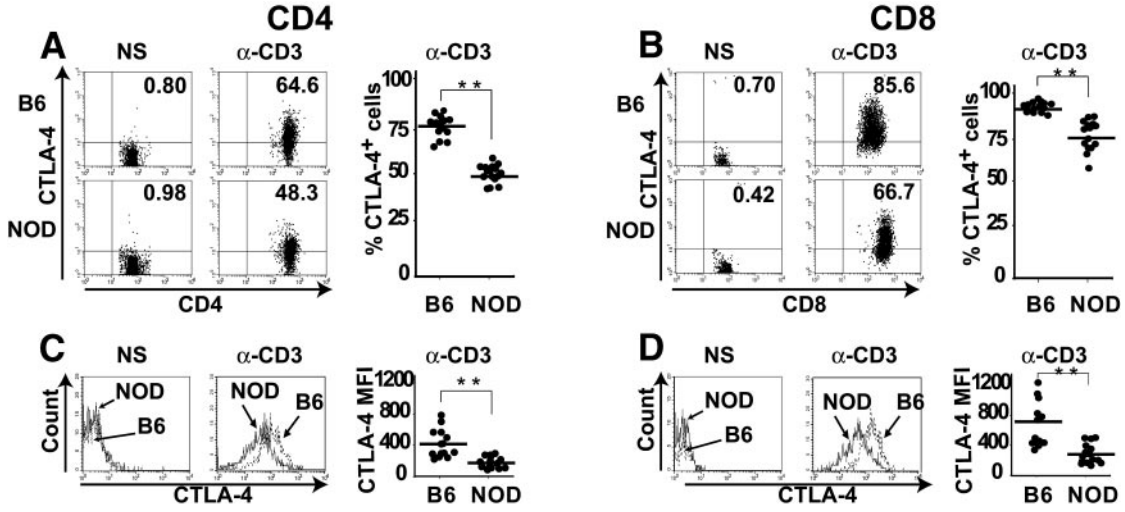

FIG. 1. CTLA-4 expression in spleen cells from NOD and B6 mice upon anti-CD3 stimulation in vitro. 2×10^6 spleen cells from NOD or B6 mice were cultured with 4 μg/ml of anti-CD3 for 48 h. Cells were then harvested, stained with monoclonal antibodies specific for CD4, CD69, CD8, or CTLA-4, and analyzed by flow cytometry. Upper panel shows representative dot plots of cell surface expression of CTLA-4 and graph with individual percentages of CTLA-4^+ cells on CD4 (*A*) and CD8 (*B*) T-cells. Lower panel shows representative histograms of intracellular expression of CTLA-4 and graph with individual mean fluorescent intensity (MFI) values of CTLA-4 intracellular expression on CD4 (*C*) and CD8 (*D*) T-cells. Nonstimulated (NS) cells were virtually devoid of $CD69^+$ cells and were gated on $CD4^+$ or $CD8^+$ cells, while activated (α-CD3) cells were gated on $CD4^+CD69^+$ or $CD8^+CD69^+$ cells. **Indicates $P < 0.001$.

FCS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, penicillin (100 units/ml), streptomycin (100 mg/ml), and 50 μmol/l 2-mercaptoethanol. Cells were stimulated with soluble anti-CD3 (4 μg/ml clone 145 2C11) alone or together with soluble anti-CD28 (5 μg/ml clone 37.51; BD Pharmingen) and/or with soluble recombinant h-IL-2 (100 units/ml; Roche). Cells were incubated for 48 h at 37°C in 5% CO_2 before being harvested and analyzed by flow cytometry (FACSCalibur; BD Biosciences) as described below.

Phenotyping. After 48 h of culture, cells were harvested and stained with anti-CD4 fluorescein isothiocyanate (FITC) (Clone H129.19), anti-CD8 Percp (Clone 53–6.7), anti-CD69 Biotin (Clone H1.2F3), and anti-CD152 (CTLA-4) phycoerythrin (PE) (clone UC10 4F10–11) or inducible T-cell costimulator (ICOS) PE (Clone 7E.17G9) in round-bottom 96-well plates. All antibodies were purchased from Pharmingen. Cells were washed in fluorescence-activated cell sorter medium (PBS containing 3% FCS and 0.05% sodium azide) and stained with anti-CD4 fluorescein isothiocyanate, anti-CD8 Percp, anti-CD152 PE, and anti-CD69 Biotin for 20 min at 4°C. Cells were washed and allophycocyanin (APC)-streptavidin conjugate was added for 20 min at 4°C. For intracellular staining, cells were first stained with anti-CD4 fluorescein isothiocyanate, anti-CD8Percp, and anti-CD69 Biotin, then washed once in fluorescence-activated cell sorter medium followed by addition of streptavidin-APC conjugate. Cells were then fixed with 1% paraformaldehyde for 30 min at room temperature and permeabilized with 0.5% saponin. After fixation and permeabilization, cells were stained with anti-CD152 PE antibody for the detection of CTLA-4 in the cytoplasm.

The amount of CTLA-4 was assessed on CD4- and CD8-activated T-cells by gating on $CD4^+CD69^+$ or $CD8^+CD69^+$ cells. The percentage of CTLA-4^+ cells in these gated populations was calculated and compared between the different mouse strains. For the measurement of intracellular expression of CTLA-4, cells were gated as described above and the median of fluorescence intensity of the CTLA-4 staining was used for comparison.

Genotyping. Genome DNA was extracted from tails according to standard techniques. A total of 196 F2 mice were genotyped using conventional PCR protocols for 119 microsatellite markers purchased from DNA Technology (Aarhus, Denmark). The genetic markers used distinguish the parental strains (B6 and NOD) by length polymorphisms in simple sequence repeats according to the Mouse Genome Informatics database. Amplification products were analyzed in ethidium bromide stained 4% agarose gels using 2% Nusieve GTG agarose (Cambrex) + 2% agarose (Sigma).

Linkage analysis. Marker positions were obtained from the published Mouse Genome Informatics map (http://www.informatics.jax.org). Genotype errors were assessed by significant deviations from the expected Mendelian inheritance or by the error-checking functions of the R/qtl software (est.rf and calc.errorlod). Analysis of quantitative trait loci (QTL) was performed using MAPMAKER/QTL 1.1 (24) and R/qtl (25). Significant logarithm of odds (LOD) scores were considered to be LOD ≥4.3 and suggestive LOD scores to be LOD ≥2.8 (26).

RNA and cDNA preparation. Cultured spleen cells were washed once with PBS and stored at −80°C. RNA was prepared from these cells using RNeasy Mini Kit (Quiagen) according to manufacturers' instructions and dissolved in 40 μl of RNase-free water followed by treatment with DNase I (Ambion). RNA concentrations were measured using a spectrophotometer, and cDNA was prepared from 300 ng total RNA using the Reverse Transcription Reagents (TaqMan) purchased from Applied Biosystems.

Real-time PCR analysis. Primers and probes used to measure the RNA expression were for the full-length CTLA-4: forward primer 5′-GGACGCAGATTTATGTCATTGATC-3′, reverse primer 5′-CCAAGCTAACTGCGACAAGGA-3′, probe 5′-(FAM)-AGAACCATGCCC-GGATTCTGACTTCC-(TAMRA)-3′; for the endogenous control genes, Acidic Ribosomal Phosphoprotein (36B4): forward primer 5′-CCCTGAAGTGCTCGACATCA-3′, reverse primer 5′-TGC-GGACACCCTCCAGAA-3′, probe 5′-(VIC)-AGAGCAGGCCCTGCACTCTCGC-(TAMRA)-3′; and for the β-actin gene: forward 5′-GGACCTGACGGACTACCTCATG-3′, reverse 5′-TCTTTGATGTCACGCACGATTT-3′, probe 5′-(VIC)-CCTGACCG-AGCGTGGCTACAGCTTC-(TAMRA)-3′. Primers were designed using Primer Express (Applied Biosystems) and purchased from Applied Biosystems. Relative expression of the transcripts was measured in the ABI prism 7900HT Sequence detection system (Applied Biosystems) and determined by relative RNA quantification using the comparative Ct method as described in the Applied Biosystems users' bulletin (27). Briefly, the amount of target transcript normalized to an endogenous control gene and relative to a calibrator sample (total RNA prepared from naïve B6 spleen cells) is given by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{endogenous\ control})$ sample in study $- (Ct_{target} - Ct_{endogenous\ control})$ calibrator.

Statistical significance. Mann-Whitney *U* and Kruskal-Wallis *H* nonparametric tests were used to compare phenotypic differences for two groups and three or more groups, respectively.

RESULTS

Defective expression of CTLA-4 in NOD cells upon anti-CD3 stimulation in vitro is controlled by gene(s) outside of the *Idd5* locus. To compare the induction of CTLA-4 expression, spleen cells from 8–10 week old B6 and NOD mice were stimulated in vitro with anti-CD3 and CTLA-4 expression in $CD4^+CD69^+$ or $CD8^+CD69^+$ cells and scored 48 h later by flow cytometry. Stainings were performed using the anti–CTLA-4 monoclonal antibody (mAb) clone, UC10-4F10-11, which recognizes the full-length but not the ligand-independent isoform of the CTLA (28). As illustrated in Fig. 1, B6 mice were found to display a higher number of CTLA-4^+ cells compared with NOD

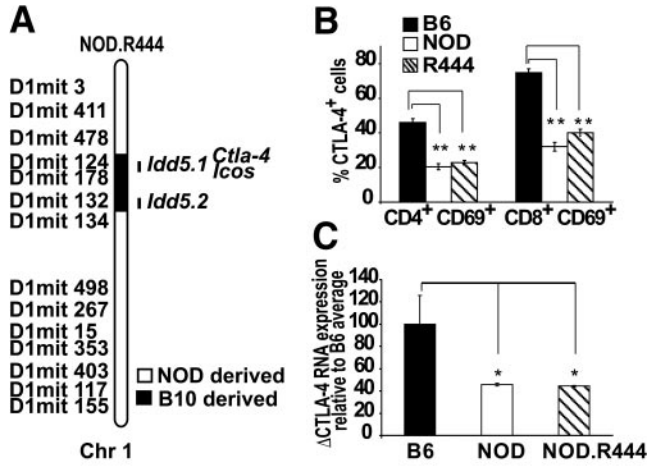

FIG. 2. The Idd5 region does not control the low CTLA-4 expression in NOD mice. Spleen cells from B6, NOD, and the NOD.R444 congenic mice were stimulated in vitro with 4 μg/ml of anti-CD3. After 48 h of culture, cells were harvested and stained with antibodies specific for CD4, CD69, CD8, and CTLA-4 and analyzed by flow cytometry. *A*: Schematic illustration of the B10 congenic region introgressed into the NOD background in the NOD.R444 congenic strain. *B*: Percentages of CTLA-4$^+$ T-cells among CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ cells after anti-CD3 stimulation of spleen cells derived from NOD, B6, or NOD.R444 mice. Bar values represent the mean of six mice ± SE. *C*: CTLA-4 RNA expression measured by real-time PCR. Total RNA was prepared from stimulated spleen cells obtained from the different mouse strains, and the amount of RNA expression was measured as $2^{-\Delta\Delta Ct}$ (see RESEARCH DESIGN AND METHODS). The percentages displayed in *C* are relative to the $2^{-\Delta\Delta Ct}$ average of B6 mice (22.62 ± 5.8). *Indicates $P < 0.05$ and **indicates $P < 0.001$.

mice. Moreover, the levels of extra cellular expression of CTLA-4 were higher in B6 cells than in NOD cells after anti-CD3 in vitro activation (Fig. 1*A* and *B*). This strain-specific difference in induction of CTLA-4 was also observed intracellularly (Fig. 1*C* and *D*) and was evident already in 3-week-old mice, i.e., before detectable insulitis (data not shown). Together, these data confirmed previous findings that NOD cells display an impaired induction of CTLA-4 expression and suggest that this deficiency is independent of an ongoing chronic insulitis (23).

The observed difference in activation-induced CTLA-4 between B6 and NOD T-cells could result from allelic differences in the *Ctla-4* gene itself. Alternatively, trans-acting genetic factors may underlie this discrepancy. To directly address this, we compared the induction of CTLA-4 in NOD congenic mice carrying the *Ctla-4* allele of B10 origin (Fig. 2*A*) (14,15). As shown in Fig. 2*B*, the R444 congenic mice displayed a proportion of CTLA-4$^+$ cells similar to the NOD parental strain when analyzing both CD4$^+$CD69$^+$ cells and CD8$^+$CD69$^+$ cells. The same pattern of expression was also observed at the RNA level for the full-length CTLA-4 isoform (Fig. 2*C*), indicating a control at the transcriptional level. Together, these data demonstrate that the genetic control of the observed difference in CTLA-4 expression is not conferred by the *Ctla-4* locus itself or by gene(s) located in the *Idd5* interval. This observation concurs with a previous report by Garchon et al. (29) suggesting that control of CTLA-4 expression is not linked to this chromosomal region.

Control of CTLA-4 expression is conferred by a major locus on chromosome 1. Having excluded the *Ctla-4* locus itself as the main locus controlling CTLA-4 expression levels, we next genetically mapped this trait. A total of 200 F2[NODxB6] mice were phenotyped by analyzing CTLA-4 expression in both CD4$^+$CD69$^+$ and

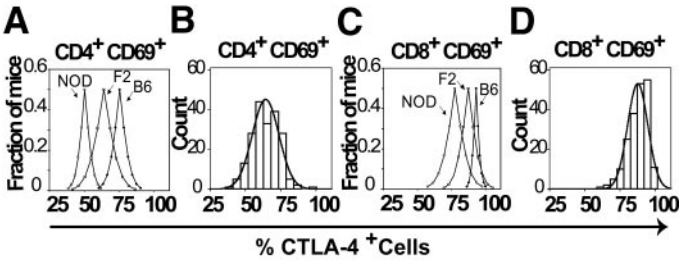

FIG. 3. Phenotypic distribution of extracellular CTLA-4 expression on CD4 and CD8 T-cells. Spleen cells from NOD and B6 parental strains and from animals of F2-cohort were stimulated in vitro with 4 μg/ml of anti-CD3 and analyzed by flow cytometry after 48 h of culture. *t*-distribution curves predicted from the analysis of CD4$^+$CD69$^+$CTLA-4$^+$ T cells (*A*) or of CD8$^+$CD69$^+$ CTLA-4$^+$ cells (*C*). Six B6, 15 NOD, and 200 F2 mice were included in this analysis. *B* and *D* illustrate the phenotypic distribution of the percentage of CTLA-4$^+$ cells for the F2 mice in comparison with the normal distribution curve of CD4$^+$CD69$^+$ (*B*) or CD8$^+$CD69$^+$ (*D*) cells.

CD8$^+$CD69$^+$ T-cells. For both of these traits, the phenotypic variance in the F2 population was larger than in the parental strains, indicating that the alleles controlling this trait were segregating in the cross (Fig. 3*A* and *C*). The percentage of CTLA-4$^+$ cells in CD4$^+$CD69$^+$ cells displayed a normal phenotypic distribution (Fig. 3*B*), suggesting that the phenotype is inherited in a quantitative fashion and is potentially affected by several loci.

Using the interval mapping method implemented in the MAPMAKER/QTL software (24), we scanned for QTLs using marker loci in all chromosomes. Taking a LOD score ≥4.3 as significant evidence in favor of linkage in accordance with the guidelines proposed by Kruglyak and Lander for genome-wide screenings (26), a region in the distal part of chromosome 1 was identified (Fig. 4*A*). This 21-cM region between D1Mit 104 and D1Mit 403 had a

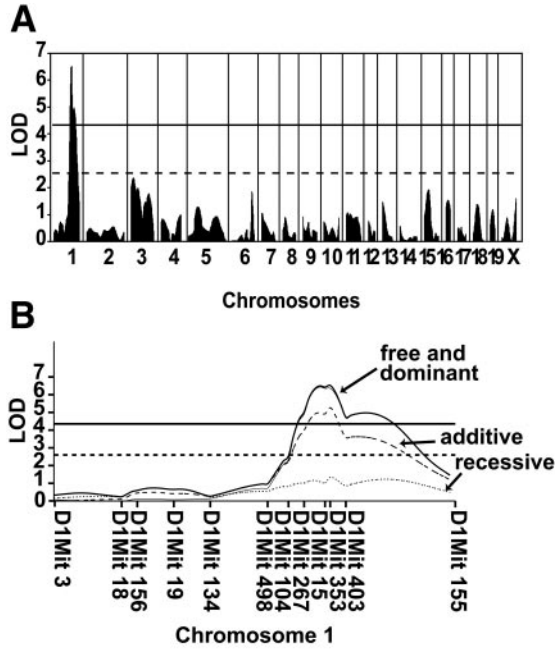

FIG. 4. Data from genome-wide scan for linkage to extracellular CTLA-4 expression on CD4$^+$CD69$^+$ T-cells upon anti-CD3 in vitro stimulation. *A*: Log-likelihood ratio plotted against genetic position in the genome. *B*: Fitness of the QTL identified on chromosome 1 to four genetic modes of action of the B6 allele is indicated. Lines indicate the thresholds for suggestive (dashed line) and significant (solid line) linkage.

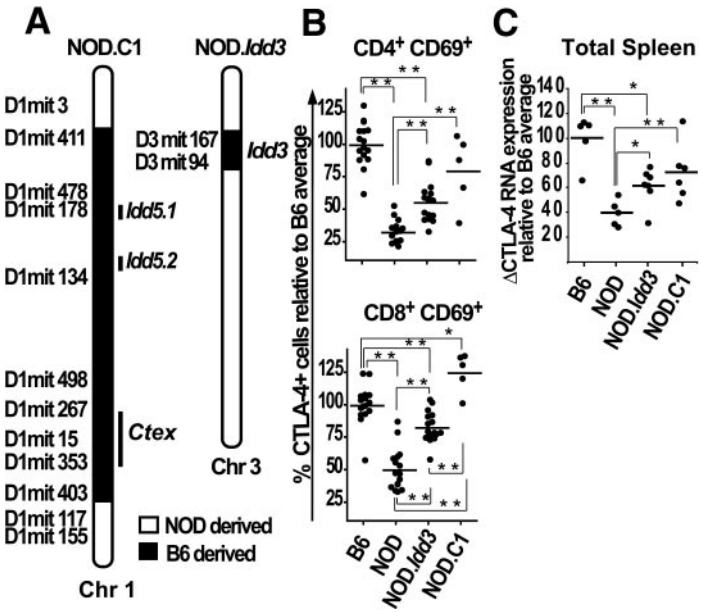

FIG. 5. CTLA-4 protein and RNA expression in different NOD congenic mouse strains upon anti-CD3 activation in vitro. Total spleen cells were stimulated with 4 μg/ml of anti-CD3 in vitro for 48 h. After culture, part of the sample was harvested for flow cytometric analysis, and the remaining cells were used to prepare RNA for real-time PCR analysis. *A*: Schematic illustration of the B6 congenic region in the NOD.C1 and the NOD.*Idd3* congenic mouse strains. *B*: CTLA-4 extracellular protein expression in $CD4^+CD69^+$ and $CD8^+CD69^+$ T-cells from B6, NOD, NOD.*Idd3*, and NOD.C1 mice. The values indicated are relative to the average of the percentage of B6 CTLA-4^+ cells for $CD4^+$ $CD69^+$ (34.8 ± 3.7%) or for $CD8^+CD69^+$ (66.4 ± 3.9%). *C*: CTLA-4 RNA expression measured by real-time PCR. Total RNA was prepared from stimulated spleen cells obtained from the different mouse strains, and the amount of RNA expression was measured as $2^{-\Delta\Delta Ct}$ (see RESEARCH DESIGN AND METHODS). The percentages displayed in *C* are relative to the $2^{-\Delta\Delta Ct}$ average of B6 mice (22.74 ± 2.21). *Indicates $P < 0.05$ and **indicates $P < 0.001$.

maximum LOD score of 6.5 close to D1Mit353 (Fig. 4*B*). The LOD score profiles recomputed under three different models of genetic action did not allow unequivocal discrimination between an additive and a dominant mode of action of the NOD allele (Fig. 4*B*). No additional chromosomal regions were identified for which evidence of linkage could meet the criteria for significant linkage. Taken together, both approaches to analyze our data showed a significant linkage to the distal part of chromosome 1 controlling the CTLA-4 expression among $CD4^+CD69^+$ cells.

In contrast to the $CD4^+CD69^+$ population, the percentage of CTLA-4^+ cells in the $CD8^+CD69^+$ cell population was found to deviate from a normal distribution (Fig. 3*D*), and to analyze this trait we therefore applied a nonparametric QTL analysis based on the R-QTL software described by Broman et al. (25). In this case, CTLA-4 expression was also found to be linked to markers in the distal part of chromosome 1 with the highest significance level close to the D1Mit353 marker (LOD = 9.8) (data not shown).

In addition, only one other region spanning the segment from D3Mit164 to D3Mit95 containing the *Idd3* locus reached a LOD score >2.0 with a maximum of 2.4 for CD4 (Fig. 4*A*) and 1.9 for CD8 cells (data not shown). Although this finding does not meet the stringent criteria suggested by Kruglyak and Lander for declaring suggestive linkage in a F2 cross, it remains interesting as a potential QTL in this region covering the *Idd3* locus. This notion is further supported by the fact that *Il-2*, the main candidate gene for the *Idd3* region on chromosome 3, has been shown to play a role in the T-cell receptor–induced expression of CTLA-4 (30).

Congenic mapping confirms that Ctex on chromosome 1 and *Idd3* on chromosome 3 contribute to the low expression of CTLA-4 in NOD. To confirm the involvement of the regions of linkage to CTLA-4 expression, we assessed the levels of CTLA-4 expression in two different congenic mouse strains. NOD.C1 is a congenic strain generated in our laboratory containing the major part of chromosome 1 of B6 origin introduced onto the NOD genetic background (Fig. 5*A*). The B6 segment spans from D1Mit411 to D1Mit403 and encompasses *Idd5.1*, *Idd5.2*, and the Ctex region found in this study to be linked to the control of CTLA-4 expression. The strain has been backcrossed five times using marker-assisted selection on chromosome 1 as illustrated in Fig. 5*A*, and it does not contain B6 alleles in any of the major *Idd* regions (data not shown). This breeding scheme does not, however, lead to the establishment of a true homozygous line, and background genetic heterogeneity might therefore contribute to the variance for CTLA-4 expression observed in the NOD.C1 strain (Fig. 5). The NOD.*Idd3* strain has been previously described and contains a B6 segment of chromosome three that spans from D3mit167 to D3Mit94 (http://jaxmice.jax.org).

Spleen cells from NOD, B6, NOD.C1, and NOD.*Idd3* mice were stimulated in vitro with anti-CD3. After 48 h of culture, cells were harvested and stained for CTLA-4 expression. As illustrated in Fig. 5, both congenic strains displayed an intermediate or fully restored CTLA-4 expression compared with the parental strains B6 and NOD. A similar pattern was observed for $CD8^+$ CTLA-4^+ cells. These data demonstrate that gene(s) located in the chromosomal regions defined by the NOD.*Idd3* and in the NOD.C1 congenic strains contribute to the low expression of CTLA-4 observed in NOD mice. In agreement with the data obtained from the genetic mapping studies, the locus on chromosome 1 plays a major role in this phenotype, while *Idd3* contributes to a lesser extent. The contribution of other loci to the control of this phenotype cannot be excluded and may underlie the increase in phenotypic variance observed in the NOD.C1 congenic mice.

We next quantified the CTLA-4 RNA expression in the parental strains and in the two congenic strains using a real-time PCR approach. As illustrated in Fig. 5*C*, the pattern of CTLA-4 full-length isoform RNA expression mimicked the data obtained from the protein expression analysis, confirming the difference between B6 and NOD at the RNA level. Moreover, it demonstrates that the B6 alleles of the *Idd3* and the *Ctex* regions can partially restore the levels of CTLA-4 RNA expression in NOD mice.

Addition of exogenous IL-2 partly restores the defective CTLA-4 expression of in vitro activated NOD T-cells. Interestingly, one of the main candidate genes for the *Idd3* region, the *Il-2* gene, has been shown to be

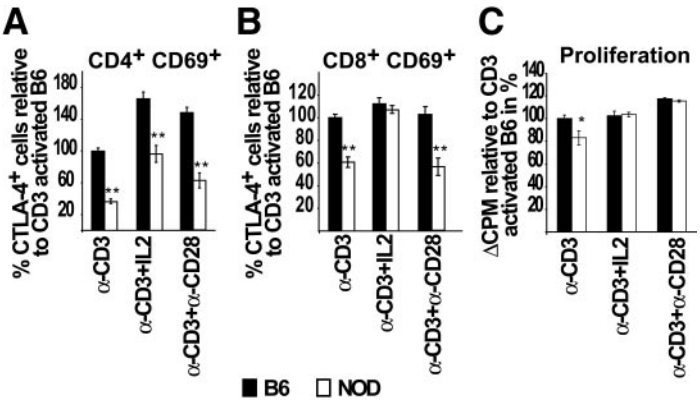

FIG. 6. IL-2 partially restores the low CTLA-4 expression in NOD mice. Spleen cells from NOD and B6 mice were stimulated in vitro with anti-CD3 alone, anti-CD3 plus rIL-2, or anti-CD3 plus anti-CD28. After 48 h of culture, cells were harvested and stained for CTLA-4, CD4, CD8, and CD69. The percentage of CTLA-4$^+$ T-cells was determined gated on CD4$^+$CD69$^+$ (*A*) and on CD8$^+$CD69$^+$ (*B*) T-cells. The values are relative to the average of the percentage of CTLA-4$^+$ cells from B6 mice cells stimulated with anti-CD3 (34.8 ± 3.8% for CD4$^+$CD69$^+$ and 66.4 ± 3.9% for CD8$^+$CD69$^+$). *C*: Proliferation of NOD and B6 spleen cells upon in vitro stimulation. Spleen cells from NOD and B6 mice were stimulated in vitro with anti-CD3 alone, anti-CD3 plus rIL-2, or anti-CD3 plus anti-CD28 for 48 h. Six hours before harvesting the cells, 5 μCi of [^{3}H]thymidine was added. The values indicated are relative to the average of cpm measured in B6 cultures stimulated with anti-CD3 ($425.39 \times 10^3 \pm 56.09 \times 10^3$). *Indicates $P < 0.05$ and **indicates $P < 0.001$.

involved in the regulation of CTLA-4 expression (30). We therefore hypothesized that allelic differences in the *Il-2* gene could underlie the observed defect of CTLA-4 induction observed in the NOD mouse. If so, excess addition of exogenous IL-2 should be able to, at least in part, restore the CTLA-4 expression levels in NOD cells. To test this hypothesis, we stimulated NOD and B6 spleen cells in vitro with anti-CD3 and added recombinant IL-2 to the cultures. Figure 6 shows that addition of exogenous rIL2 or anti-CD28 mAb in NOD and B6 α-CD3–stimulated cultures augment the percentage of CTLA-4$^+$ cells when gated on CD4$^+$CD69$^+$ cells, and that rIL-2 is more potent than anti-CD28 mAbs. Contrary to CD4 cells, the percentages of CTLA-4$^+$ cells gated on CD8$^+$CD69$^+$ cells did not increase significantly in B6 cultures by adding rIL2 or anti-CD28 mAb. On the other hand, addition of rIL2 in NOD cultures increases the percentage of CTLA-4$^+$ cells in the CD8 population to the levels found in B6 cultures, which was not observed with addition of anti-CD28 mAb. Thus, rIL2 is more efficient in driving CTLA-4 expression than anti-CD28 mAb, but it only restores the phenotype on NOD CD8 and not on CD4 cells. This may be explained by peculiarities in the activation and the role of CTLA-4 on these two populations (31,32)

Interestingly, IL-2 and CD28 costimulation was similarly effective in mediating CD3-induced T-cell proliferation in cells of B6 as well as NOD origin (Fig. 6*C*). Thus, while signals through CD28 costimulation fail to mediate CTLA-4 expression to the same levels in NOD as in B6 T-cells, it is equally able to activate proliferation in T-cells of both strains.

Separate control of aberrant CTLA-4 and ICOS expression in NOD T-cells. In parallel with lower levels of CTLA-4 expression, activated T-cells of NOD origin have been reported to express higher levels of ICOS (16). The differential expression of ICOS in NOD and control strains was suggested to be directly controlled by either polymorphisms in the *Icos* gene or, alternatively, by other molecules genetically regulated by the *Idd5.1* gene. A possibility suggested by these authors is that the high ICOS levels are an indirect effect of low expression levels of the liCtla-4 isoform in NOD T-cells. Since we had identified the major control of CTLA-4 expression levels to be mediated by *Ctex* and *Idd3*, we next determined the ICOS expression levels in the congenic strains used for analyzing CTLA-4 expression. As illustrated in Fig. 7, activated T-cells of NOD origin expressed significantly higher levels of ICOS compared with T-cells of B6 origin. In congenic mice carrying only the *Idd5.1/2* loci (R444) or both the *Idd5.1/2* and *Ctex* loci (NOD.C1), ICOS expression levels were similar to those observed in B6. On the other hand, ICOS expression levels in NOD.*Idd3* congenic mice remained similar to those observed in NOD T-cells. In contrast to CTLA-4 expression, we conclude that ICOS expression differences are controlled by the *Idd5* region and not significantly affected by the *Idd3* or the *Ctex* loci.

DISCUSSION

The *Ctla-4* gene is located in the *Idd5* susceptibility region on chromosome 1 that affects the development of diabetes and insulitis in NOD mice (33–35). Previously, congenic studies have shown that the interaction of two Idds in this chromosomal region, *Idd5.1* and *Idd5.2*, confers susceptibility to diabetes (14). These findings and recent reports narrowing down *Idd5.1* to a 1.2-Mb region (15) support the notion of *Ctla-4* as a putative candidate gene in this chromosomal region. The functional role of the NOD allele of the *Ctla-4* gene as an etiological factor in the pathogenesis of diabetes however remains unclear. Full-length CTLA-4 is clearly aberrantly expressed in activated T-cells as demonstrated previously (23) and confirmed in this study. Moreover, blocking of CTLA-4 with an antibody against the full-length protein has been demonstrated to increase diabetes incidence in NOD mice (21,36), favoring the notion that CTLA-4 expression levels are of importance to diabetes pathogenesis. However, since NOD.*Idd5* congenic mice display a decrease in diabetes incidence despite expressing similar levels of the CTLA-4 full-length isoform as NOD mice, it would appear that additional

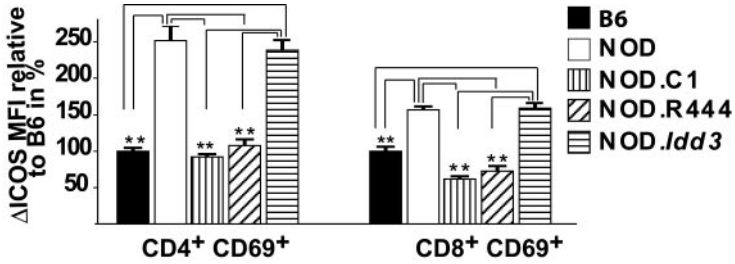

FIG. 7. ICOS expression on T-cells of congenic mice after in vitro stimulation. Spleen cells from NOD, B6, NOD.C1, NOD.R444, and NOD.Idd3 were cultured in the presence of 4 μg/ml of anti-CD3 plus 100 units/ml of rIL-2 for 48 h. Stimulated cells were harvested and stained for CD4, CD8, CD69, and ICOS expression. ICOS expression was measured as mean fluorescence intensity (MFI) on activated CD69$^+$CD4$^+$ and CD69$^+$CD8$^+$ cells. Indicated bar values represent the mean values for each strain relative to the average of MFI values in CD4$^+$CD69$^+$ (22.85 ± 1.17) or CD8$^+$CD69$^+$ (31.01 ± 1.83) T-cells derived from B6 mice. **Indicates $P < 0.001$.

effects of the *Idd5* region may play a role in NOD pathogenesis. Such an effect has been suggested by Wicker and colleagues (10,15,28), demonstrating that the susceptibility of diabetes conferred by the *Idd5* locus is associated with a splice form encoding a molecule lacking the CD80/CD86 ligand-binding domain, referred to as the ligand-independent isoform (liCTLA-4).

It has previously been noted that the control of defective levels of activation-induced CTLA-4 in NOD T-cells is not primarily controlled by the structural gene itself (29); however, the locus conferring this trait has not been genetically mapped. Combining a genome-wide scanning approach with congenic mapping, we found that a novel locus named *Ctex*, defined as a 21-cM region spanning from markers D1Mit104 to D1mit403 in the distal part of chromosome 1, and a region on chromosome 3 overlapping with *Idd3/Il-2* region act in concert to mediate this effect.

The Ctex locus was identified as a 21-cM region located 40 cM from the *Ctla-4* structural gene. Thus, the NOD.R444 mice express levels of CTLA-4 after activation similar to NOD mice while the NOD.C1 congenic mouse strain containing a larger B6 segment of chromosome 1 restored the CTLA-4 expression to levels similar to those found in B6 cells. This places a major locus controlling this trait in the region in the distal part of chromosome 1 that is defined by the recombination sites of the NOD.C1 and NOD.R444 congenic mice. Interestingly, this region also overlaps the NOD mouse lupus susceptibility gene *Babs2/Bana3* (37), the QTLs for NKT-cell numbers (38), and IgG serum levels (39). Moreover, recently Zuchelli et al. (40) reported that genes in this chromosomal region influenced the defective central tolerance ascribed to NOD mice. This is particularly intriguing since these authors also reported that similar to the observation here, a second major region contributing to this phenotype was identified on chromosome 3. The chromosomal region identified on chromosome 1 contains around 200 genes, making it difficult to evaluate potential candidates. While further analysis of subcongenic mice is ongoing to better define positional candidate genes for this trait, we note that the candidates in this interval include the *Cd3z*, *Pdcd1*, and *Bcl-2*, although the latter two map slightly outside of the one LOD drop region in the linkage analysis.

The linkage of control of CTLA-4 expression levels to the *Idd3/Il-2* locus is not totally surprising given that IL-2 has previously been shown to play an important role in the expression of CTLA-4 upon T-cell activation (30). In naïve T-cells, the majority of CTLA-4 is found in intracellular vesicles (41) and upon T-cell activation is released to the cell surface and migrates to the site of T-cell receptor contact with the APC (42,43). Induction of CTLA-4 following T-cell activation is shown to depend on signals mediated by IL-2. Thus, IL-2–deficient mice do not induce any detectable surface expression of CTLA-4 upon anti-CD3 activation (30), and these mice show a polyclonal activation of lymphocyte and severe hemolytic anemia (44–46). Moreover, IL-2 has been shown to be required for survival and function of regulatory T-cells, which constitutively express CTLA-4 and seem to mediate regulation through this molecule (47–50). Allelic variation in the IL-2 gene has been demonstrated to be associated with an altered glycosylation pattern of the molecule, and the NOD allele has been suggested to constitute a plausible etiological factor corresponding to the *Idd3* diabetes susceptibility locus (51–53). However, attempts to correlate these allelic differences with the ability of the IL-2 molecule to mediate induction of proliferation have failed, and no evidence of functional implications of the NOD allele has until now been unequivocally demonstrated. In fact, IL-2 of B6 and NOD origin have been previously demonstrated to be equally capable of sustaining the proliferation of an IL-2–dependent cell line in vitro (51). In view of this, it is particularly interesting that the addition of IL-2 could in part overcome the difference of CTLA-4 expression between B6 and NOD observed after in vitro activation of splenic T-cells, while costimulation by CD28 ligation had only a marginal effect. This observation suggests that the NOD allele of IL-2 may functionally differ from the B6 allele in its ability to direct the transcription of activation-induced CTLA-4, while retaining a normal proliferation-inducing capacity. Further analyses of the downstream events of IL-2 receptor signaling under those conditions are ongoing to address this issue.

The previously reported observation that ICOS expression levels vary between activated NOD versus B6 T-cells was replicated here. Moreover, the fact that this expression difference appears to be solely controlled by the *Idd5* locus suggests that expression is independent of the genetic control expression of the CTLA-4 full-length isoform. Whether ICOS expression is controlled by polymorphisms in the *Icos* gene itself or dependent on the differential expression of the liCTLA-4 isoform observed in NOD T-cells remains to be determined.

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