The Soluble CTLA-4 Splice Variant Protects From Type 1 Diabetes and Potentiates Regulatory T-Cell Function

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OBJECTIVE—*CTLA4* gene variation associates with multiple autoimmune disorders, including type 1 diabetes. The *CTLA4* susceptibility allele was found to generate decreased levels of mRNA encoding soluble CTLA-4 (sCTLA-4) relative to the full-length isoform, the functional consequence of which is as yet unknown. In this study, we investigated the contribution of sCTLA-4 to immune regulation with the aim to elucidate the functional basis of the disease association of *CTLA4*.

RESEARCH DESIGN AND METHODS—To model the disease-associated splicing variation of *CTLA4*, we generated NOD mice in which sCTLA-4 mRNA is silenced by RNA interference.

RESULTS—We found that loss of sCTLA-4 impairs the function of regulatory T (Treg) cells. This functional defect could be attributed, at least in part, to the failure of sCTLA-4 knockdown (KD) Treg cells to downregulate dendritic cell costimulation. sCTLA-4 KD Treg cells, in contrast with wild-type Treg cells, failed to inhibit colitis induced by transfer of $CD4^+CD45RB^{hi}$ cells into NOD.SCID animals. Furthermore, diminished sCTLA-4 expression accelerated the onset of autoimmune diabetes in transgenic mice.

CONCLUSIONS—Our results demonstrate that sCTLA-4 participates in immune regulation by potentiating the function of Treg cells. The functional outcome of silencing this splice variant in the NOD model provides an explanation for the association of *CTLA4* variation with autoimmunity. Lower sCTLA-4 expression from the susceptibility allele may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Our unprecedented approach establishes the feasibility of modeling splicing variations relevant to autoimmunity. ***Diabetes* 60:1955–1963, 2011**

Susceptibility to type 1 diabetes is modulated by >40 genomic loci (1). Several likely causal genes, including *CTLA4*, have been identified (1–4). *CTLA4* polymorphism associates with multiple autoimmune disorders in addition to type 1 diabetes (4,5). Yet the functional contribution to autoimmunity of the disease-associated *CTLA4* allele remains unknown. The critical role of this gene in immune regulation is well established and is best exemplified by the phenotype of *Ctla4* knockout (KO) mice that succumb to severe lymphoproliferation within a few weeks of birth (6,7). Similarly, the specific deletion of *Ctla4* in $Foxp3^+$ regulatory T (Treg) cells alone disrupts immune regulation and causes lethal lymphoproliferation (8). CTLA-4 has been suggested to fulfill its inhibitory function by several distinct mechanisms. Its homology to the costimulatory molecule CD28 may allow CTLA-4 to sequester CD80 and CD86 that provide positive signals to T cells via CD28 (9). CTLA-4 has also been shown to directly elicit negative signals that counteract T-cell activation (10–13). In addition, CTLA-4 was found to prevent T lymphocytes from building lasting interactions with antigen-presenting cells (APCs) (14). Notably, CTLA-4 was shown to act both in a cell-autonomous and a non–cell-autonomous manner to modulate T-cell responses (15).

Understanding the exact function of CTLA-4 has been a long-standing challenge whose complexity further increased with the discovery of alternative splice variants (4,16,17). *Ctla4* encodes a transcript with four exons. Splicing generates a full-length transcript (flCTLA-4) and two shorter transcripts that skip exon 2 (ligand-binding domain) or exon 3 (transmembrane domain). These shorter mRNAs are translated into proteins termed "ligand-independent" CTLA-4 (liCTLA-4, present in mouse but not in human) (4,18) and "soluble" CTLA-4 (sCTLA-4, present in both human and mouse) (4,16,17), respectively.

The diabetes-associated *CTLA4* susceptibility allele has been correlated to a decrease in sCTLA-4 mRNA relative to the full-length transcript (4,19). Because the respective function of these splice variants has not been resolved, the consequence of a change in splicing frequency is unclear. We decided to study the function of sCTLA-4 to understand how decreased levels of this molecule might affect immune regulation. Our aim was to determine whether a change in sCTLA-4 levels could be causal for the disease association of *CTLA4* in humans. To this end, we chose to specifically examine sCTLA-4 function within the context of the NOD mouse model for type 1 diabetes (20). *Ctla4* KO mice were generated by deletion of exons 2 and 3, ablating expression of all three isoforms (6,7). Because of overlapping exon use between CTLA-4 splice variants, a conventional KO approach cannot be used to delete either of the shorter isoforms without also affecting flCTLA-4. Instead, we exploited the posttranscriptional silencing mechanism of RNA interference (RNAi) to target sCTLA-4 while retaining wild-type (WT) levels of both flCTLA-4 and liCTLA-4. We generated transgenic NOD mice in which only sCTLA-4 is silenced by RNAi and found that loss of this splice variant alone reduced the potency of Treg cells. Loss of sCTLA-4 accelerated type 1 diabetes onset, supporting a causal role for the splicing variation associated with autoimmunity in humans. Together, our results demonstrate a significant role for sCTLA-4 in immune regulation.

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RESEARCH DESIGN AND METHODS

Mice. WT, transgenic, and congenic NOD mice were bred and maintained at the University of Würzburg in accordance with institutional guidelines. All experiments were approved by the university animal care committee. Lentiviral transgenic mice were generated by microinjection of single-cell NOD embryos as described previously (21).

Lentiviral construct for shRNA expression. The pLB vector (21) was modified by replacing the U6-shRNA-CMV-GFP cassette with a CMV-GFP-mir30shRNA cassette (from pPRIME, 22). pLB was digested with *Eco*RI, and the overhangs were blunted before digesting the vector with *Xba*I. The products of the digest were separated by gel electrophoresis, and the backbone was extracted from the gel and purified. The CMV-GFP-mir30shRNA cassette was cut out from pPRIME by *Xba*I/PmeI double-digest, gel purified as above, and ligated into the pLB backbone to generate the pLBM vector. shRNA constructs were cloned into pLBM using the restriction sites *Xho*I and *Eco*RI located in the mir30 cassette. Positive clones were identified by colony PCR and verified by sequencing. Lentiviral particles were generated as described previously (21), and high-titer preparations ($>10^8$ infectious particles/mL) were used for NOD embryo injection.

Validation of shRNA potency by luciferase reporter assay. The cDNA for flCTLA-4, sCTLA-4, and liCTLA-4 (gift from Dr. Vijay Kuchroo, Harvard Medical School) were cloned into the 3′ UTR of the Renilla luciferase gene in the psiCheck2 vector (Promega, Madison, WI). pLBMs containing a control shRNA or shRNA sequences against the exon 2–exon 4 junction sequence of sCTLA-4 were cotransfected with the psiCheck2 reporter constructs into 293FT cells. Cells were lysed 48 h after transfection, and luciferase activities were measured in a Fluostar Optima luminometer (BMG Labtech, Offenburg, Germany). Renilla luciferase activity was normalized to Firefly luciferase activity that is also expressed from the psiCheck2 plasmid to adjust for transfection efficiency. Renilla luciferase activity of control shRNA transfected cells was used as a baseline value (100% relative luminescence). Results from duplicate transfections expressed in percent relative luminescence are shown. The most efficient and specific shRNA target sequence, used for transgenesis, was GCAGATTTATGTCATTGCTAAA.

Quantitation of CTLA-4 mRNA levels ex vivo. WT and sCTLA-4 knockdown (KD) mice were each injected with 5 μg anti-CD3 antibody intravenously. Six hours later, the animals were killed and dissected, and the spleen was immediately homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA). Extracted RNA was reverse-transcribed, and mRNA levels for sCTLA-4, liCTLA-4, and flCTLA-4 were quantitated by Taqman PCR using the following forward primer, reverse primer, and probe, respectively: sCTLA-4, acccaccgccatactttgtg/aggacttcttttctttagcaatgacat/aatctgcgtcccgttgcccatg; liCTLA-4, gccttttgtagccctgctca/tcagaatccgggcatggtt/ttcttttcatcccagtcttctctgaagatcca; and flCTLA-4, actcatgtacccaccgccata/gggcatggttctggatcaat/catgggcaacgggacgcagatttat.

Flow cytometry. All flow cytometry measurements were performed on a FACSCanto II flow cytometer (BD Biosciences, Bedford, MA), and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Cell purification. Cell populations used in culture were magnetically isolated using MACS beads (Miltenyi Biotec, Cologne, Germany) for negative selection of $CD4^+$ cells and positive selection of $CD25^+$, $CD62L^+$, or $CD11c^+$ cells according to the manufacturer's instructions.

Proliferation assays. [^{3}H]thymidine (0.5 μCi/well) was added to cultures for the last 16 h, and incorporation was measured by scintillation measurement using a microbeta-counter (Perkin-Elmer, Foster City, CA). Results are expressed in counts per minute.

Regulatory T-cell assays. T-cell subsets were purified from lymph nodes and spleen by magnetic separation using a $CD4^+CD25^+$ isolation kit (Miltenyi Biotech) according to the manufacturer's instructions. CD4-depleted cells from WT mice were irradiated (20 Gy) and used as APC (4×10^5/well). $CD4^+CD25^-$ Teff cells (2.5×10^4/well) were mixed with $CD4^+CD25^+$ Treg cells at the indicated ratios and stimulated with 1 μg/mL anti-CD3 antibody in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine, 2-mercaptoethanol, sodium pyruvate, and HEPES (all from Gibco/Invitrogen, Carlsbad, CA).

Cytokine measurements. Cytokine concentrations in culture supernatants were measured by ELISA (eBioscience, San Diego, CA) and Cytometric Bead Array (BD Biosciences) according to the manufacturers' instructions.

Dendritic cell coculture assay. Spleens were digested with Liberase Blendzyme 2 (Roche, Mannheim, Germany), and splenic dendritic cells (DCs) were purified by magnetic separation using anti-CD11c beads (Miltenyi Biotech) according to the manufacturer's instructions. DCs (4×10^4/well) were cultured with $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (1.6×10^5/well), as indicated, in the presence of 1 μg/mL anti-CD3 antibody. T cells were stained with CFSE (Invitrogen) before culture, and DCs ($CD11c^+CFSE^-$) were analyzed for CD86 expression by flow cytometry after 40 h of culture.

Colitis induction. WT $CD4^+CD45RB^{hi}$ T cells (4×10^5) were injected alone or with $CD4^+CD25^+$ T cells (10^5) from WT or sCTLA-4 KD NOD mice into NOD.SCID recipients. Mice were killed after 8–9 weeks, and paraffin-embedded colon sections were stained with hematoxylin–eosin and scored for disease in a blind fashion on a scale from 0 to 4 (23): grade 0 = no changes; grade 1 = minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2 = mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3 = mild to moderate inflammatory cell infiltrates that are sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4 = marked inflammatory cell infiltrates that are often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion.

Statistical analyses. All statistical analyses were performed using the Prism software package (GraphPad Software Inc., La Jolla, CA). Survival curves were compared using the log-rank test. All other comparisons were performed by unpaired *t* test.

RESULTS

Generation of sCTLA-4 KD NOD mice. To investigate the role of sCTLA-4 in immune function and autoimmunity, we set out to generate NOD mice lacking this splice variant. A KO approach is not suited to ablating a single transcript composed of only exons that are also present in other splice variants of the same gene. Because RNAi is a posttranscriptional event, it is more amenable to targeting individual splice variants, and we therefore assessed the feasibility of silencing sCTLA-4 in an isoform-specific manner using short-hairpin RNA (shRNA) constructs. To achieve specificity, we designed shRNA sequences that span the exon 2–exon 4 junction unique to the transcript encoding sCTLA-4 (Fig. 1*A*). Functional validation of several sequences identified one shRNA construct capable of targeting sCTLA-4 mRNA for degradation without affecting either flCTLA-4 or liCTLA-4 (Fig. 1*B*). Silencing was highly specific for the sCTLA-4 variant despite a high degree of sequence identity between the target region and flCTLA-4 (86% identity, Supplementary Fig. 1). We used this construct to generate transgenic NOD mice by perivitelline injection of lentivirus into NOD zygotes (21). The founder line used for all subsequent experiments was determined to contain a single copy of the lentiviral transgene (Supplementary Fig. 2). Transgene expression was detected in 75% of cells on average (Fig. 1*C*), and expression was stable within individual mice and throughout generations. Quantitative PCR measurements confirmed that sCTLA-4 mRNA was significantly reduced in splenocytes from transgenic mice (Fig. 1*D*). In contrast, both flCTLA-4 and liCTLA-4 levels were comparable to those of control animals.

sCTLA-4 silencing diminishes the suppressive activity of Treg cells in vitro. Initial characterization of lymphoid organs from sCTLA-4 KD mice showed that T-cell development was unaffected (Fig. 2*A* and *B*). Neither thymic nor peripheral T-lymphocyte populations differed in phenotype or frequency between WT and sCTLA-4 KD mice. T lymphocytes from sCTLA-4 KD mice also proliferated similarly to their WT counterpart in response to in vitro stimulation (Fig. 2*C*). Further characterization of $CD4^+$ subsets showed that $Foxp3^+$ Treg cells were normal in both their frequency and their expression of Foxp3, CD25, total CTLA-4, and GITR (Fig. 3).

Several recent reports have highlighted the role of CTLA-4 in the function of Treg cells (8,23–25). Although the assumption until now has been that flCTLA-4 is the major isoform contributing to overall CTLA-4 function, we wanted to investigate whether sCTLA-4 participates in the suppressive function of Treg cells. Treg cells from sCTLA-4

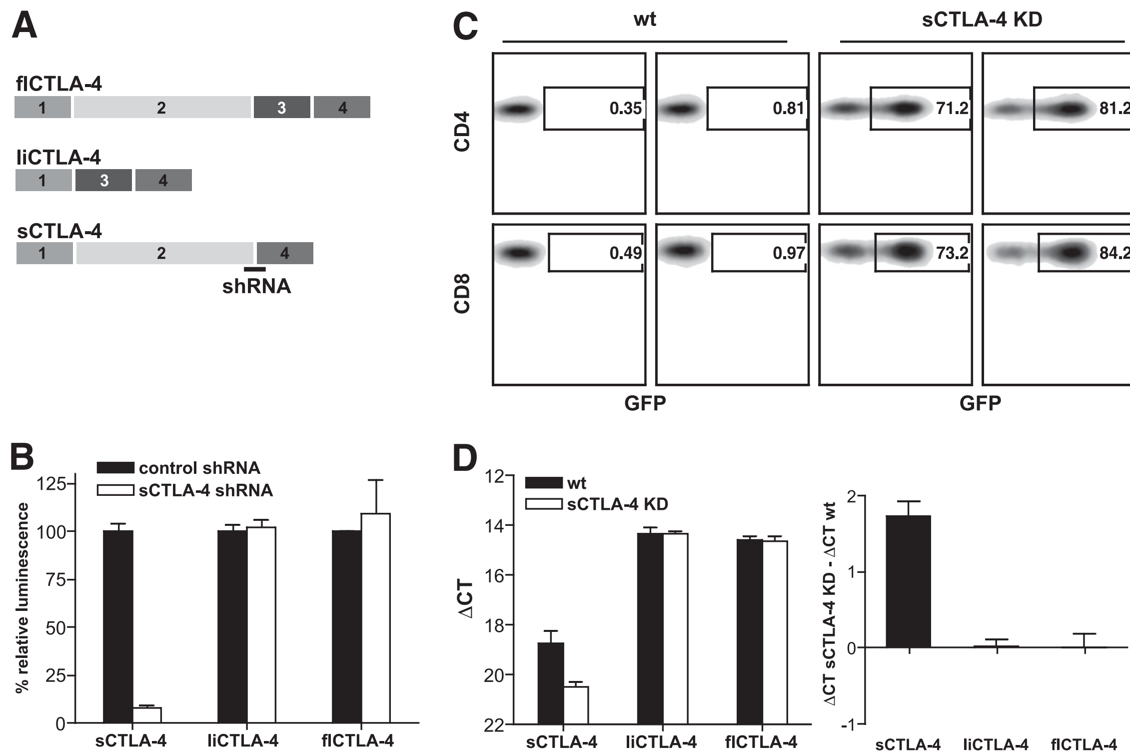

FIG. 1. Generation of sCTLA-4 KD NOD mice. ***A*****: Schematic representation of the exon structure of the flCTLA-4, liCTLA-4, and sCTLA-4 mRNAs. The position of the shRNA target sequence is indicated.** ***B*****: The potency and specificity of sCTLA-4 silencing were validated in a luciferase reporter assay, where shRNA efficiency was tested individually on sCTLA-4, liCTLA-4, and flCTLA-4 reporter constructs. Representative results of four similar experiments are shown.** ***C*****: sCTLA-4 KD mice were generated by lentiviral transduction of NOD embryos with a vector containing GFP, and transgene expression was measured by flow cytometry. Expression in CD4$^+$ and CD8$^+$ T cells from two representative WT and sCTLA-4 KD mice is shown. The transgene was bred to homozygosity to improve expression.** ***D*****: mRNA levels for all three CTLA-4 isoforms were measured in splenocyte lysates prepared 6 h after intravenous anti-CD3 antibody injection (5 μg/mouse) to increase overall CTLA-4 expression.** ***n*** **= 3 mice for both groups,** ***P*** **= 0.0353 for sCTLA-4, with a reduction of ~70% (1.74 cycles). Representative results of three similar experiments are shown.**

KD and WT mice were equally potent in vitro when used at a 1:1 ratio of effector T cells (Teff) to Treg cells. However, at higher ratios, suppression by transgenic Treg cells was weaker compared with that effected by WT cells. At ratios of 5:1 (Teff:Treg) or higher, inhibition of Teff cell proliferation and interferon-γ production was significantly dampened when Treg cells from sCTLA-4 KD mice were used (Fig. 4*A* and *B*). Similar results were observed when Teff cells from sCTLA-4 KD mice were used (Supplementary Fig. 3). Loss of sCTLA-4 is apparently sufficient to impair the suppressive function of Treg cells. This partial defect could have a developmental origin, or alternatively, sCTLA-4 could directly contribute to the effector function of Treg cells. To distinguish between these two possibilities, we generated induced-Treg cells (iTreg) (26). The in vitro differentiation of these cells can be assumed to bypass potential defects in thymic Treg cell development. CD4$^+$CD62L$^+$ T cells were cultured in the presence of transforming growth factor (TGF)-β, and their differentiation into iTreg cells was assessed by intracellular Foxp3 measurement. Naïve T lymphocytes from WT and sCTLA-4 KD NOD mice could be differentiated into iTreg cells with similar efficiencies, with ~80–85% of CD4$^+$ cells expressing Foxp3 after TGF-β treatment (Fig. 4*C*). The suppressive capacity of iTreg cells derived from sCTLA-4 KD mice was again decreased at higher Teff:Treg ratios (Fig. 4*D*), directly implicating sCTLA-4 in the effector mechanism of Treg cells.

Treg cell–derived sCTLA-4 modulates APC costimulation. CTLA-4 is thought to perform its function, at least in part, by binding to costimulatory molecules on the surface of APCs (9–13). The exact mechanism by which such binding translates into inhibition of T-cell activation is unclear. One possibility pertinent to the function of Treg cells is the direct downmodulation of these molecules on the surface of APC (8,27). Wing et al. (8) have shown that *Ctla4*-deficient Treg cells fail to counter the upregulation of costimulatory molecules on DCs in culture. However, the respective contributions of flCTLA-4 and sCTLA-4 to this phenomenon have not been investigated. To test whether sCTLA-4 participates in the modulation of APC costimulation, we cultured DCs with Treg cells from either WT or sCTLA-4 KD mice, in the presence or absence of Teff cells. Consistent with previous reports (8,24,27), the presence of Treg cells from WT mice diminished CD86 expression on the DC surface. In contrast, sCTLA-4 KD Treg cells were only partially capable of inhibiting CD86 expression (Fig. 5), suggesting a role for Treg cell–derived sCTLA-4 in modulating the costimulatory potential of APCs.

Loss of sCTLA-4 impairs Treg cell activity in vivo. Having established that the loss of sCTLA-4 affects the suppressive activity of Treg cells in vitro, we wanted to address whether the defects observed in vitro directly affect immune regulation in vivo. CTLA-4 has been shown to be critical to the function of Treg cells within a colitis model where disease is induced by transfer of CD4$^+$CD45RB^{hi} T cells and can be suppressed by the cotransfer of CD4$^+$CD25$^+$ T cells (23,28). Although this colitis model is not usually used in conjunction with the NOD mouse strain, one earlier study reported colitis induction

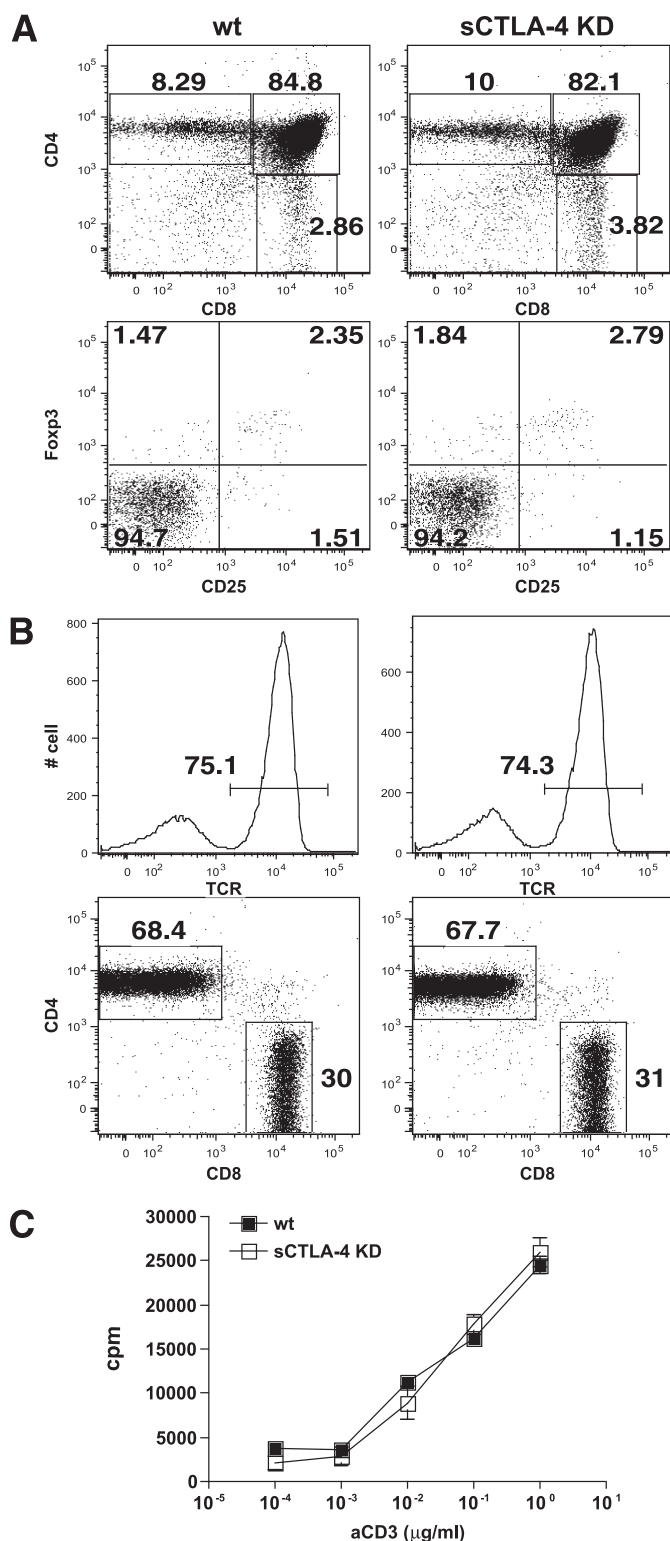

FIG. 2. T-cell development is not affected by sCTLA-4 silencing. *A*: Representative analysis of WT and sCTLA-4 KD thymocytes stained with anti-CD4, anti-CD8, anti-CD25, and anti-Foxp3 antibody. *Top*: Total thymocytes. *Bottom*: Gated on $CD4^+CD8^-$ (CD4SP) cells. *B*: Representative analysis of WT and sCTLA-4 KD lymph node cells stained with anti-TCRβ, anti-CD4, and anti-CD8 antibody. *Top*: All lymph node cells. *Bottom*: Gated on $TCR\beta^+$ cells. *C*: Lymph node cells from WT and sCTLA-4 KD mice were stimulated with anti-CD3 antibody, and proliferation was measured after 72 h. Results are representative of three similar experiments; error bars indicate SEM.

after the transfer of $CD4^+CD45RB^{hi}$ NOD T cells into NOD. SCID animals (29). We hypothesized that the defect in Treg cell function caused by the loss of sCTLA-4 may be apparent in this model, because overall CTLA-4 blockade had been shown to impair the suppressive activity of $CD4^+CD25^+$ cells in the same context (28). The transfer of $CD4^+CD45RB^{hi}$ cells into NOD.SCID recipients resulted in mild but significant pathology (Fig. 6). The simultaneous transfer of WT Treg cells markedly reduced colon inflammation. In contrast, the cotransfer of sCTLA-4 KD Treg cells failed to inhibit pathogenic $CD4^+CD45RB^{hi}$ cells and resulted in pathology comparable to that observed in recipients of $CD4^+CD45RB^{hi}$ cells alone. These data implicate sCTLA-4 in Treg cell function in vivo. Results from these experiments therefore support the notion that sCTLA-4 expression modulates immune reactivity by contributing to the suppressive function of Treg cells.

sCTLA-4 silencing accelerates diabetes onset. The NOD mouse strain is the most widely used animal model for type 1 diabetes (20). The rationale for targeting sCTLA-4 in the NOD model was to replicate the human disease-associated *CTLA4* polymorphism. We hypothesized that if the splicing difference observed in humans is causal for the diabetes association of this gene, sCTLA-4 silencing would increase disease frequency in NOD mice. Because most NOD female mice develop diabetes, we anticipated that an increase in disease may not be discernible in the fully susceptible background. We therefore also bred the sCTLA-4 KD transgene onto the protected *Idd5.1* congenic background (30). We chose this particular congenic line because the effect of the *Idd5.1* locus is due to a protective allele of *Ctla4* that generates higher liCTLA-4 levels (30,31). The protection is independent of a change in sCTLA-4 itself, yet allows the evaluation of sCTLA-4 function within the context of a protective *Ctla4* allele. We therefore sought to investigate whether the loss of sCTLA-4 could diminish the protective effect of this allele. We found that sCTLA-4 silencing had no significant effect in the fully susceptible NOD background (Fig. 7*A*). Female sCTLA-4 KD NOD mice developed diabetes with a frequency and onset kinetics comparable to those of the WT NOD cohort. However, although the increase in disease frequency caused by sCTLA-4 knockdown in *Idd5.1* mice did not reach significance (Fig. 7*A*), gene silencing fully reverted the delay in diabetes onset effected by the protective *Idd5.1* allele (Fig. 7*B*). These results provide the first functional evidence that loss of sCTLA-4 increases susceptibility to type 1 diabetes.

DISCUSSION

The most recent genome-wide association study of type 1 diabetes, combined in a meta-analysis with two previous similar studies, reported no fewer than 41 distinct genomic loci associated with disease (1). Among the likely causal gene variants identified to date are several genes known to play a pivotal role in immune regulation, such as *IL2*, *IL2RA*, and *CTLA4*. Yet, each of these genes makes only a modest contribution to disease risk (1,3). Most disease gene variants are indeed characterized by seemingly mild alterations in gene expression or function (4,32,33). For example, although CTLA-4 function is known to be critical in mice (6,7), the human diabetes-associated *CTLA4* susceptibility allele differs from the protective allele only in its splicing frequency (4,19). It is difficult to ascribe causality to such subtle variations, particularly when their functional consequences are unknown. The respective function

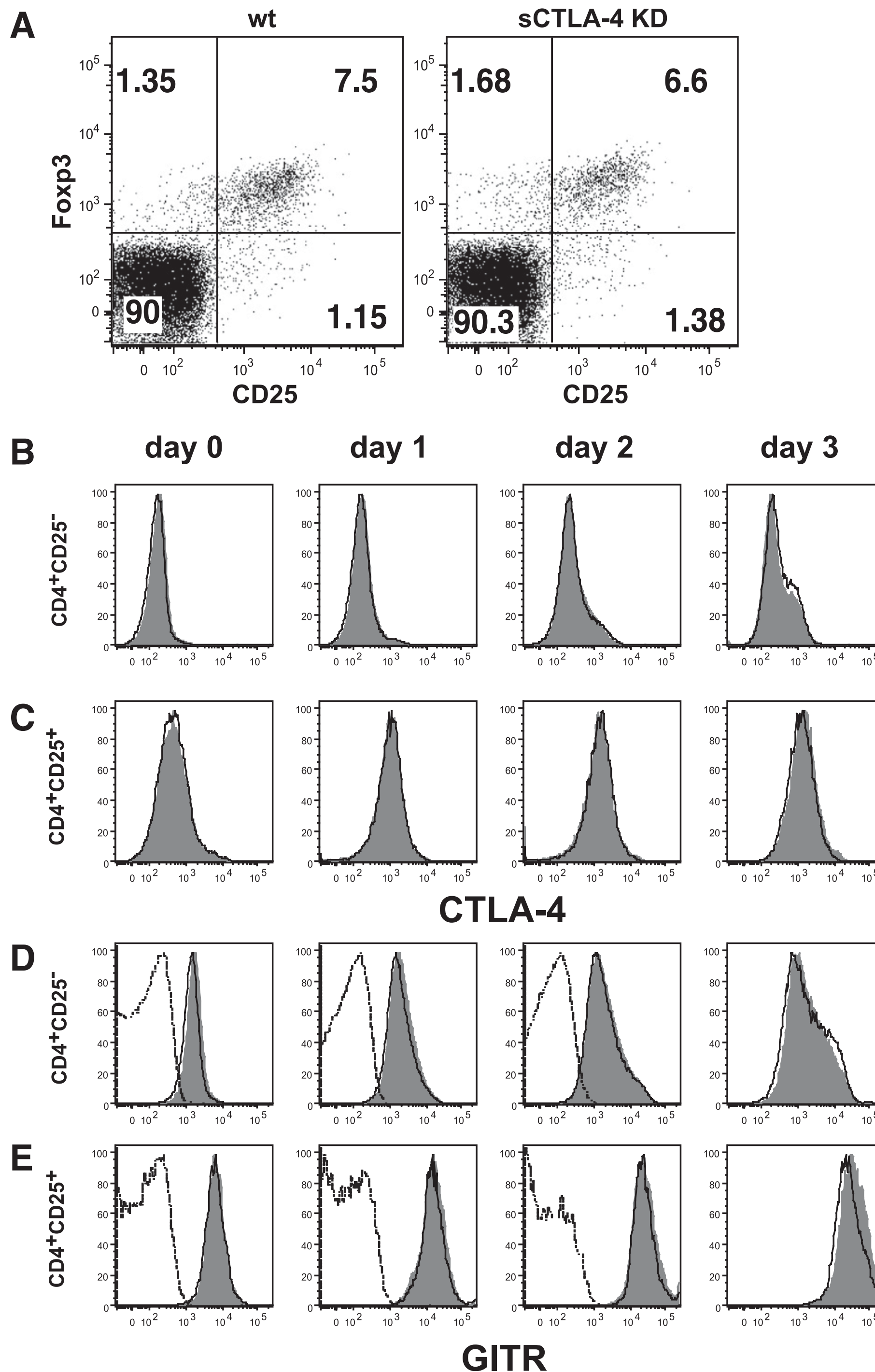

FIG. 3. Treg cell frequency and cell surface markers are not perturbed by loss of sCTLA-4. ***A:*** **CD25 and Foxp3 expression in TCR⁺CD4⁺ lymph node cells from WT and sCTLA-4 KD mice.** ***B–E*****: Purified T-cell subpopulations were stimulated with irradiated splenocytes and anti-CD3 antibody, and expression profiles were analyzed at the indicated times. CTLA-4 expression (intracellular staining) (*B* and *C*) and GITR expression (*D* and *E*) in CD4⁺CD25⁻ cells (*B* and *D*) and CD4⁺CD25⁺ cells (*C* and *E*) from WT (black line) and sCTLA-4 KD (filled histogram) mice are shown.**

of the different *CTLA4* splice variants has not been resolved to date. Consequently, it was unclear until now whether the shift in *CTLA4* splicing frequency is causal for disease association and how this change in splicing may functionally affect immune regulation.

Modeling subtle gene variations, and splicing variations in particular, poses a significant technical challenge that cannot be approached with conventional methods. To overcome this issue, we pioneered the use of isoform-specific RNAi in the mouse. We generated transgenic NOD mice in which a single splice variant of *Ctla4*, namely, sCTLA-4, is silenced by RNAi, and were thereby able to model the *CTLA4* gene variation associated with type 1 diabetes in humans. Our findings using this novel approach demonstrate that loss of sCTLA-4 increases disease susceptibility and suggest that the splicing change caused by the disease-associated *CTLA4* allele in humans may directly affect immune regulation.

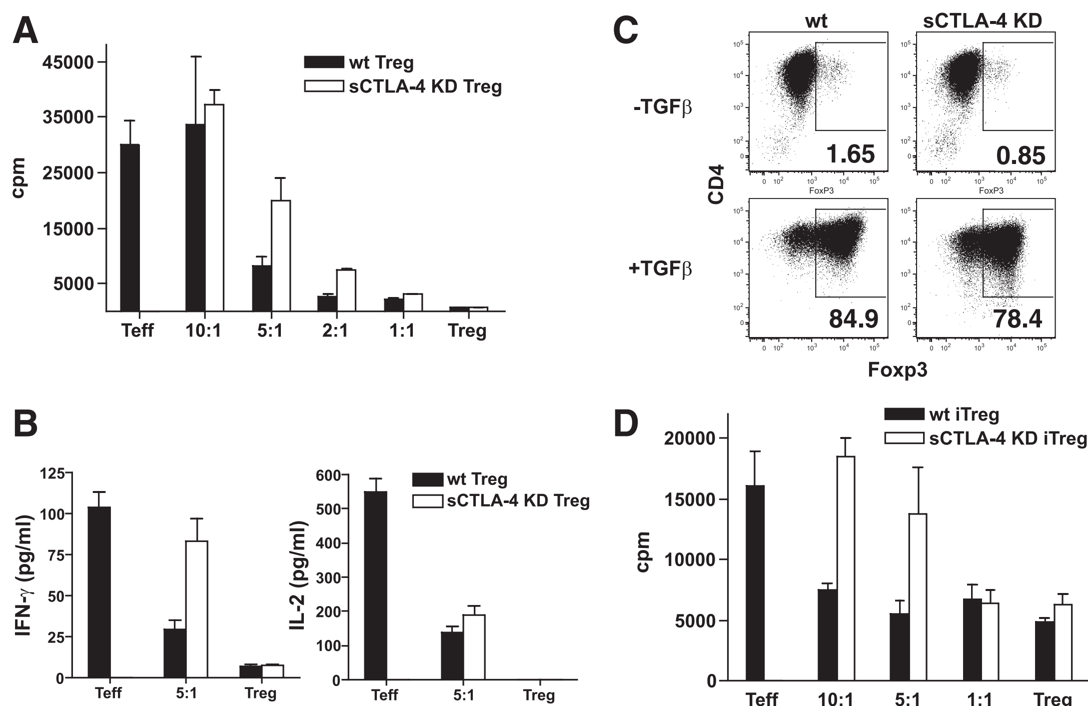

FIG. 4. sCTLA-4 silencing diminishes the suppressive activity of Treg cells in vitro. ***A*** **and** ***B*****: WT CD4$^+$CD25$^-$ Teff cells and WT or sCTLA-4 KD CD4$^+$CD25$^+$ Treg cells were stimulated with irradiated splenocytes and anti-CD3 antibody at the indicated cell ratios. Proliferation (*A*) or cytokine production (*B*) was measured after 72 h. *A*: P = 0.0003 and P = 0.05 at the 2:1 and 5:1 ratios, respectively. *B*: P = 0.0243 for interferon-γ at the 5:1 ratio. *C* and *D*: CD4$^+$CD62L$^+$ T cells from WT or sCTLA-4 KD mice were stimulated with anti-CD3 and anti-CD28 antibody in the presence or absence of TGF-β (2 ng/mL) for 96 h, and Foxp3 expression was measured (*C*). Freshly isolated WT CD4$^+$CD25$^-$ Teff cells and TGF-β–treated cells (iTreg) were then stimulated with irradiated splenocytes and anti-CD3 antibody at the indicated cell ratios (*D*), and proliferation was measured at 72 h. *D*: P = 0.0027 at the 10:1 ratio. All data are representative of three or more experiments. Error bars indicate SEM.**

In addition, our results demonstrate a role for sCTLA-4 in Treg cell function. Treg cells were shown to depend on CTLA-4 ~10 years ago (28,34). Several more recent studies have further highlighted the reliance of Treg cells on CTLA-4 as an effector of immune regulation (8,23–25,35). Read et al. (23,28) showed in a colitis model that CTLA-4 blockade was sufficient to abrogate protection from disease by Treg cells. Notably, their work demonstrated that CTLA-4 antibody acted directly on Treg cells, and that anti-CTLA-4 Fab fragments were sufficient to inhibit Treg cell function (23). Similarly, You et al. (35) showed that CTLA-4 blockade accelerated disease in NOD mice and that this effect was dependent on the presence of Treg cells. These results are consistent with our findings that sCTLA-4 contributes to Treg cell effector mechanisms, as shown by the partial defect of iTreg cells derived from sCTLA-4 KD mice and the reduced suppressive capacity of sCTLA-4 KD Treg cells both in vitro and in vivo. Studies using both antibody blockade and cells from *Ctla4*-deficient mice showed that CTLA-4 expressed by Treg cells is involved in the modulation of APC costimulation (8,36). Our data using Treg cells from sCTLA-4 KD mice suggest that this function may be fulfilled in part by the soluble isoform of CTLA-4. Recent work by Qureshi et al. (37) has demonstrated that flCTLA-4, owing to its capacity to bind and internalize CD80 and CD86, can substantially deplete costimulatory molecules from the APC surface. The relative contributions of the soluble and full-length CTLA-4 isoforms to the mechanisms by which *CTLA4* regulates T-cell responses in a cell-extrinsic manner therefore remain to be clarified. Notably, the partial defect we observed when using sCTLA-4 KD Treg cells to inhibit Teff cell proliferation in vitro mirrors that described by Wing et al. (8) in the context of Treg cells with a *Foxp3*-dependent *Ctla4* deletion. In vivo data from Wing et al. (8) support the notion that a complete loss of *Ctla4* in the Treg cell compartment has much more severe consequences than the mere loss of sCTLA-4. Nevertheless, sCTLA-4 KD and *Ctla4*-deficient Treg cells display similarities, particularly in vitro, implying a significant role for sCTLA-4 in overall CTLA-4 function.

Because the loss of sCTLA-4 alone compromises immune regulation, it is likely that its function is distinct from and not redundant with that of flCTLA-4. In the context of autoimmune diabetes, the effect of sCTLA-4 silencing was only detectable in combination with the protective *Idd5.1* allele derived from the B10 background that generates higher amounts of liCTLA-4 (18,31). The liCTLA-4 splice variant has been proposed to regulate the activation threshold of effector/memory T cells in a cell-autonomous manner (18). We speculate that sCTLA-4, which likely acts cell-extrinsically, only significantly affects the diabetogenic T-cell response when effector T cells are not already sensitized by the NOD susceptibility allele at *Idd5.1*. The hyperreactivity of effector T cells related to liCTLA-4 deficiency in the NOD background may overshadow the partial defect in Treg cell function caused by sCTLA-4 silencing. Restoring liCTLA-4 levels with the protective *Idd5.1* allele in NOD *Idd5.1* congenic mice may then in turn reveal the functional consequence of sCTLA-4 silencing. Of note, the human *CTLA4* gene does not generate a ligand-independent splice variant (4). In the complete absence of liCTLA-4, it is plausible that the function of sCTLA-4 in humans may be more critical in the regulation of T-cell responses than can be inferred from the mouse model where liCTLA-4 also contributes to *Ctla4*-mediated regulation.

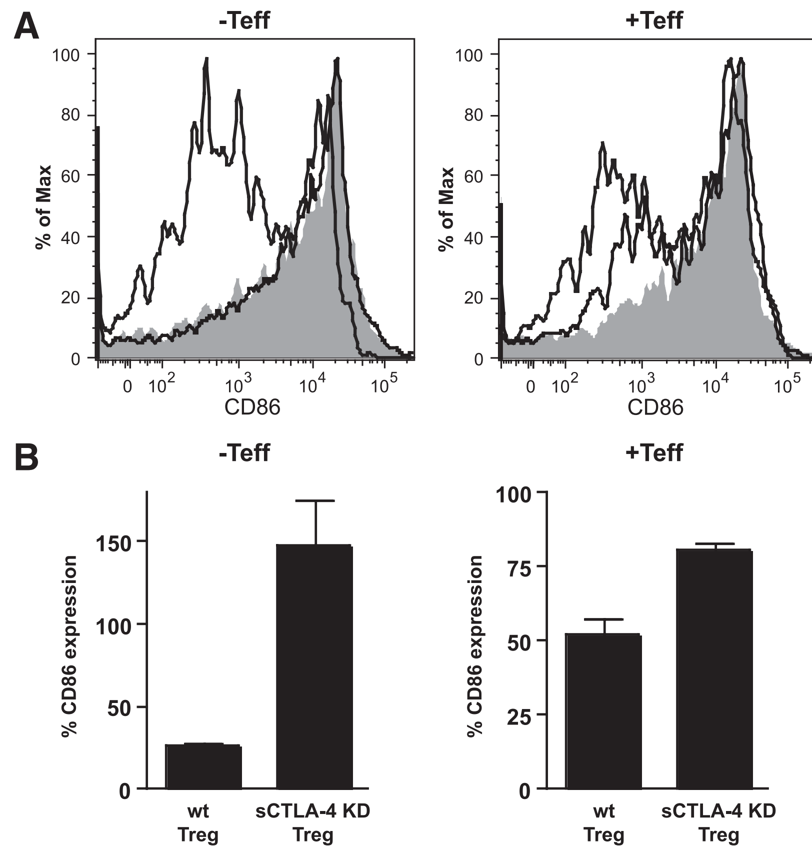

FIG. 5. Treg cell–derived sCTLA-4 modulates APC costimulation. Splenic CD11c+ DCs from WT mice were cultured alone (filled histogram), with WT CD4+CD25+ Treg cells (gray line), or with sCTLA-4 KD CD4+CD25+ Treg cells (black line) in the presence or absence of WT CD4+CD25− Teff cells. CD86 expression was measured on CD11c+ cells after 40 h. *A*: Representative flow cytometry histograms. *B*: CD86 expression on DCs cocultured with Treg cells relative to that of DCs cultured alone (combined results from two experiments are shown). P = 0.0494 and P = 0.041 for DCs with Treg cells only and Treg cells with Teff cells, respectively.

None of the most recent studies investigating CTLA-4 function (8,15,23–25,37) have addressed the potential contribution of the minor splice isoforms of CTLA-4. The technical hurdles involved and the lack of adequate reagents available are obvious deterrents to more detailed functional analyses. But it is clear that further studies are required to determine exactly which roles the individual CTLA-4 splice variants fulfill, particularly if we

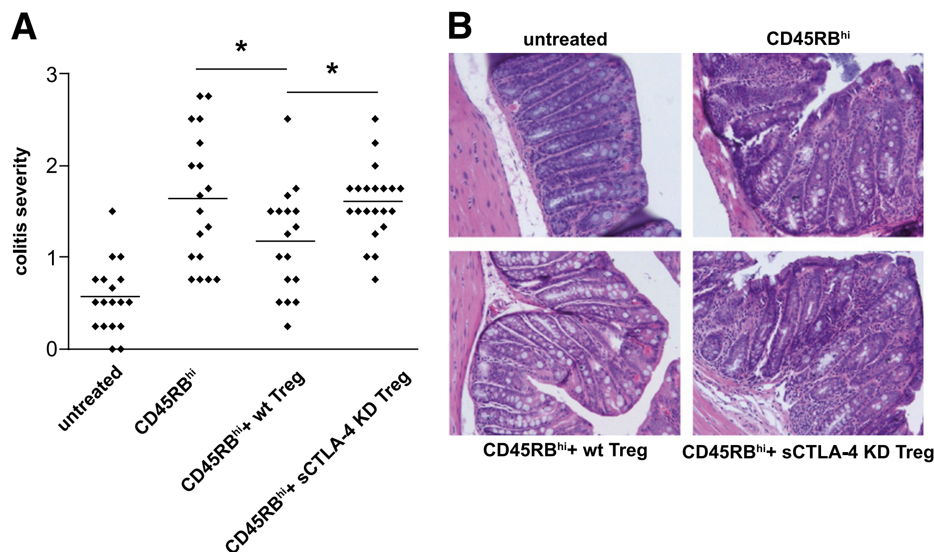

FIG. 6. Loss of sCTLA-4 impairs Treg cell activity in vivo. NOD.SCID mice were injected with CD4+CD45RBhi T cells from WT NOD animals, with or without CD4+CD25+ Treg cells from WT or sCTLA-4 KD mice. *A*: Individual colitis scores (averaged from four histologic sections per mouse) from two independent experiments (n = 17–21 per group). CD45RBhi vs. CD45RBhi+wt Treg P = 0.045. CD45RBhi+wt Treg vs. CD45RBhi+sCTLA-4 KD Treg: P = 0.0116. CD45RBhi vs. CD45RBhi+sCTLA-4 KD Treg: P = 0.83. *Significant P values (P < 0.05). *B*: Representative histology for colon sections from all four groups. (A high-quality digital representation of this figure is available in the online issue.)

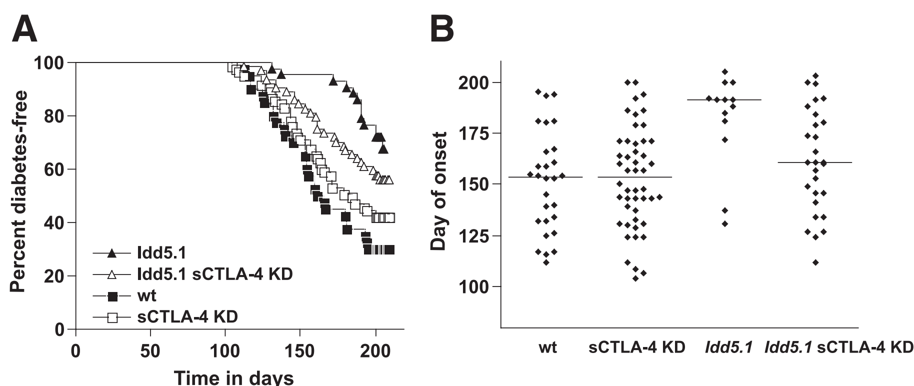

FIG. 7. sCTLA-4 silencing accelerates diabetes onset. ***A*****: Female WT (*n* = 40), sCTLA-4 KD (*n* = 83), *Idd5.1* (*n* = 43), and *Idd5.1* sCTLA-4 KD (*n* = 64) NOD mice were tested for diabetes by weekly measurement of glycosuria. Survival proportions are shown. Log-rank test: WT vs. sCTLA-4 KD *P* = 0.16; WT vs. *Idd5.1 P* < 0.0001; WT vs. *Idd5.1* sCTLA-4 KD *P* = 0.0026; sCTLA-4 KD vs. *Idd5.1 P* = 0.0009; sCTLA-4 KD vs. *Idd5.1* sCTLA-4 KD *P* = 0.0547; *Idd5.1* vs. *Idd5.1* sCTLA-4 KD *P* = 0.0893.** ***B*****: The day of onset for all diabetic mice is shown. Lines indicate median day of onset. WT *n* = 28; sCTLA-4 KD *n* = 48; *Idd5.1 n* = 13, *Idd5.1* sCTLA-4 KD *n* = 28. *t* test: WT vs. sCTLA-4 KD *P* = 0.77; WT vs. *Idd5.1 P* = 0.0006; WT vs. *Idd5.1* sCTLA-4 KD *P* = 0.14; sCTLA-4 KD vs. *Idd5.1 P* = 0.0004; sCTLA-4 KD vs. *Idd5.1* sCTLA-4 KD *P* = 0.16; *Idd5.1* vs. *Idd5.1* sCTLA-4 KD *P* = 0.0186.**

are to understand how *CTLA4* regulates T-cell activation at both a cell-intrinsic and a cell-extrinsic level (15).

In conclusion, we have investigated the function of the soluble splice variant of CTLA-4 with the aim to explain the association of *CTLA4* variation with autoimmunity in humans. We found that loss of sCTLA-4 impairs the function of Treg cells and compromises immune regulation, with the notable consequence of increasing the risk of autoimmunity in the NOD background. Our study provides the first evidence for a causal relationship between the autoimmunity-associated splicing variation of *CTLA4* and the disease risk. Last, our work underscores the utility of RNAi to study the function of single splice variants in vivo. Work by Wang et al. (38) revealed that >90% of human genes are subject to alternative splicing. In light of this report and the likely contribution of alternative splicing to phenotypic variability, the novel approach we have described will prove useful for future studies aiming to investigate disease-associated gene variants.

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