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 mouse 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+CD45RBhi 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.

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 C6l4 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 (fCTLA-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 (lCTLA-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). C6l4 KO mice were generated by deletion of exons 2 and 3, ablatting 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 fCTLA-4. Instead, we exploited the posttranscriptional silencing mechanism of RNA interference (RNAi) to target sCTLA-4 while retaining wild-type (WT) levels of both fCTLA-4 and lCTLA-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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Loss of sCTLA-4 Impairs Regulatory T-cell Function

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.

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 EcoRI, and the shRNAs were blunt-ended before digesting the vector with XhoI. The products of the digestion were separated by gel electrophoresis, and the shRNA was extracted from gel and purified. The CMV-GFP-mir30shRNA cassette was subsequently incorporated by XhoI into the plB backbone and used for transgenesis.

Flow cytometry. 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 using MACS beads (Miltenyi Biotec, Cologne, Germany) for negative selection.

Validation of shRNA potency by luciferase reporter assay. The pLBM vector (21) was modified by replacing the GFP coding sequence with the Firefly luciferase (Fluc) gene to adjust for transfection efficiency. The Fluc expression vector was cotransfected with psiCheck2 plasmid to adjust for transfection efficiency, and luciferase activities were measured in a Fluostar Optima luminometer (BMG Labtech, Offenburg, Germany). Renilla luciferase activity was measured by using a microbeta-counter (Perkin-Elmer, Foster City, CA). Results are expressed in counts per minute.

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 isofrom-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. 1A). Functional validation of several sequences identified one shRNA construct capable of targeting sCTLA-4 mRNA for degradation without affecting either fctLA-4 or liCTLA-4 (Fig. 1B). Silencing was highly specific for the sCTLA-4 variant despite a high degree of sequence identity between the target region and fctLA-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. 1C), 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. 1D). 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 NOD mice showed that T-cell development was unaffected (Fig. 2A 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. 2C). 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 fCTLA-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 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. 4A 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. 4C). The suppressive capacity of iTreg cells derived from sCTLA-4 KD mice was again decreased at higher Teff:Treg ratios (Fig. 4D), 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 thymic Treg cell development. CD4+CD62L+ T cells were cultured 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. 4C). The suppressive capacity of iTreg cells derived from sCTLA-4 KD mice was again decreased at higher Teff:Treg ratios (Fig. 4D), directly implicating sCTLA-4 in the effector mechanism of Treg cells.

**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+CD45RBhi T cells and can be suppressed by the coinjection 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...
after the transfer of CD4+CD45RBhi 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+CD45RBhi 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+CD45RBhi cells and resulted in pathology comparable to that observed in recipients of CD4+CD45RBhi 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. 7A). 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. 7A), gene silencing fully reverted the delay in diabetes onset effected by the protective Idd5.1 allele (Fig. 7B). 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
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. 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.
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). 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 fCTLA-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 Sakaguchi et al. in the context of Treg cells with a Foxp3-dependent CtlA4 deletion (8). In vivo data from Sakaguchi et al. support the notion that a complete loss of CtlA4 in the Treg cell compartment has 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 fCTLA-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 immunoregulation.
T-cell responses than can be inferred from the mouse model where liCTLA-4 also contributes to Ctla4-mediated regulation. 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

![Figure 5](image1.png)

**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.

![Figure 6](image2.png)

**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. B: Representative histology for colon sections from all four groups.
CTL4 splice variants fulfill, particularly if we 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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L.S.W. and S.K. conceived the project. K.D.G., P.Z., D.B.R., and S.K. performed experiments. K.D.G., P.Z., D.B.R., A.Z., L.S.W., and S.K. analyzed data. S.K. supervised the project and wrote the manuscript. All authors commented on and edited the manuscript.

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