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The B7-Independent Isoform of CTLA-4 Functions To Regulate Autoimmune Diabetes

Melanie Stumpf,¹ Xuyu Zhou,² and Jeffrey A. Bluestone

The critical role of CTLA-4 in inhibiting Ag-driven T cell responses upon engagement with its ligands, B7-1 and B7-2 and its importance for peripheral T cell tolerance and T cell homeostasis has been studied intensively. The CTLA-4 splice variant ligand-independent (li)-CTLA-4 is expressed in naive and activated T cells and can actively alter T cell signaling despite its lack of a B7 binding domain. To study the effect of li-CTLA-4 in regulating T cell responses in the context of autoimmunity, we engineered a B6. CTLA-4 (floxed-Exon2)-BAC-transgene, resulting in selective expression of li-CTLA-4 upon Cre-mediated deletion of Exon 2. Introducing the B6.BAC into the NOD background, which is genetically deficient for li-CTLA-4, restores mRNA levels of li-CTLA-4 to those observed in C57BL/6 mice. Furthermore, re-expressing this ligand nonbinding isoform in NOD mice reduced IFN-γ production in T effector cells accompanied by a significant decrease in insulitis and type 1 diabetes frequency. However, selective expression of li-CTLA-4 could not fully rescue the CTLA-4 knockout disease phenotype when bred onto NOD.BDC2.5.CTLA-4 knockout background because of the requirement of the full-length, B7-binding CTLA-4 molecule on T effector cells. Thus, the li-CTLA-4 form, when expressed at physiologic levels in the CTLA-4–sufficient NOD background can suppress autoimmunity; however, the functionality of the li-CTLA-4 isoform depends on the presence of the full-length molecule to alter effector T cell signaling. The Journal of Immunology, 2013, 190: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; BAC, bacterial artificial chromosome; fi, full-length; KO, knockout; li, ligand-independent; pancLN, pancreatic lymph node; T1D, type 1 diabetes; Treg, regulatory T; WT, wild-type.

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he negative regulator CTLA-4 has been established to critically affect T cell function and peripheral tolerance (1–3). In humans, the CTLA-4 gene has been reported to contribute to a general susceptibility for autoimmune diseases, especially for endocrine disorders but also systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis (4–6). CTLA-4 deficiency in mice results in a severe form of lymphoproliferative disease accompanied by multiorgan infiltration and death within 2–5 wk after birth (7, 8). The inhibitory function of CTLA-4 is most obvious upon Ag-specific T cell activation, resulting in decreased T cell proliferation and cytokine production, consequently diminishing the T cell response (9–11). Several mechanisms of CTLA-4–mediated suppression have been proposed, such as competition of CTLA-4 with CD28 for binding to their shared ligands B7-1 and B7-2 (1, 12–14). In addition, it has been suggested that accumulation of CTLA-4 within the immunological synapse disrupts CD28 localization therein (15–17). Both mechanisms of action have been described to be dependent on the extracellular domain of CTLA-4 (1, 15). Thus, many efforts have focused on this ligand interaction to address CTLA-4 function in controlling T cell responses. However, multiple studies have described an additional mechanism for CTLA-4 inhibitory function mediated by its intracellular domain. We and others have shown that the biochemical basis for CTLA-4 function is associated with the interaction of the tyrosine phosphatases, protein phosphatase 2 (PP2A) and Src homology 2 (SH2) domain-containing protein tyrosine phosphatase (SHP-2) (12, 18–21), with the cytoplasmic tail of CTLA-4 and that this interaction promotes dephosphorylation of the TCR chain as well as other TCR complex components like linker for activation of T cells and ZAP70 (22, 23). Moreover, CTLA-4 was demonstrated to inhibit ERK phosphorylation/activation as well as c-JNK and therefore additionally regulates signaling members of the MAPK family (24–26). These results describe a mechanism of action by which CTLA-4 can block TCR proximal and distal signaling and consequently attenuates cell cycle progression, cytokine production, and proliferation through its cytoplasmic tail. Interestingly, recent work by Singer et al. (27) indeed showed that TCR hyposignaling requires CTLA-4’s internal domain but also demonstrated that this can occur in a costimulatory/B7-independent fashion. In addition, we have found that CTLA-4 within lipid rafts is able to control TCR signaling events in the absence of B7 engagement (S. Chikuma and J.A. Bluestone, unpublished observations). Most significantly in this context is the recent discovery of a naturally occurring CTLA-4 isoform (ligand-independent [li]-CTLA-4), which acts in a B7 li fashion (28). Kuchroo and colleagues (29) reported that overexpression of li-CTLA-4 in T cells reduces proliferation, cytokine production and TCRζ phosphorylation in CTLA-4 knockout (KO) T cells transfected with li-CTLA-4. This isoform of CTLA-4 has been associated with type 1 diabetes (T1D) in humans and the NOD mouse model (28, 30, 31). In the latter, the CTLA-4 gene was mapped within the T1D susceptibility locus idd5.1 (32, 33), and recent studies suggested that linkage between disease and the CTLA-4 gene is primarily based on a significant reduction in li-CTLA-4, since the NOD Idd5.1 interval produces ~70% less li-CTLA-4 than does the C57BL/6 (B6) allele (28). The
molecular basis for this observation has been suggested to a single nucleotide polymorphism in Exon 2 of the CTLA-4 gene (28), resulting in altered splicing favoring the inclusion of Exon 2, which encodes the extracellular domain of the protein. Recently, Kuchroo’s and Wicker’s groups showed that constitutive expression of only li-CTLA-4 resulted in reduced NOD diabetes frequency (34). However, the use of constitutive promoters results in nonphysiological transgene expression levels that may have had nonphysiological effects, especially considering the differential expression of li-CTLA-4 among specific T cell subsets and upon T cell activation (29, 35, 36). Thus, we aimed to study the role of li-CTLA-4 in the context of autoimmune diabetes by changing the physiologic levels of the isoform in NOD mice to B6 levels. In addition, we examined how small changes in levels of the li-CTLA-4 isoform in the presence or absence of full length (fl)-CTLA-4 altered T cell function.

The B6.CTLA-4.BAC was bred onto the NOD background under conditions that delete Exon 2, resulting in B6-level expression of the li-CTLA-4 isoform. Increased expression of li-CTLA-4 to B6 levels significantly reduced T1D incidence in NOD mice. Further analyses on mice bred on the NOD.BDC2.5-CTLA-4-KO background showed that protection from T1D development requires fl-CTLA-4 expression on T effector versus T regulatory (Treg) cells. Our results clearly demonstrate the importance of li-CTLA-4 in controlling T1D development in the NOD mouse and describe, to our knowledge for the first time, that this effect strongly depends on the presence of an fl molecule.

Materials and Methods

Generation of NOD.B6-CTLA-4–Exon 2 deleting bacterial artificial chromosome transgenic mice

A B6.BAC (RP24-316c5) containing exclusively the CTLA-4 locus was engineered to express loxP sequences upstream and downstream of the Exon 2 of the CTLA-4 gene, which encodes the entire extracellular domain. The modified bacterial artificial chromosome (BAC) was purified using cesium chloride gradient ultracentrifugation and microinjected into pronuclei of NOD mouse embryos. Founder mice carrying the B6.CTLA-4/foxed-Exon 2)-BAC-transgene were crossed to the VAV-Cre deleter strain (termed NOD-Tg-Cre mice) (37). Vav1-Cre transgenic mice were a gift from Dr. D. Kioussis (Division of Molecular Immunology, MRC National Institute for Medical Research, London, U.K.). In some studies, the mice were further bred onto the NOD.CTLA-4 KO or NOD.BDC2.5-CTLA-4 KO strains, which were generated and maintained in our laboratory (7). All mice were backcrossed and maintained on the NOD background. Mice were housed in a specific pathogen-free facility at the University of California San Francisco Transgenic/Targeted Mutagenesis Core Facility into pronuclei of NOD mouse embryos. Founder mice carrying the B6.CTLA-4/foxed-Exon 2)-BAC-transgene were crossed to the VAV-Cre deleter strain (termed NOD-Tg-Cre mice) (37). Vav1-Cre transgenic mice were a gift from Dr. D. Kioussis (Division of Molecular Immunology, MRC National Institute for Medical Research, London, U.K.). In some studies, the mice were further bred onto the NOD.CTLA-4 KO or NOD.BDC2.5-CTLA-4 KO strains, which were generated and maintained in our laboratory (7). All mice were backcrossed and maintained on the NOD background. Mice were housed in a specific pathogen-free facility at the University of California San Francisco Transgenic/Targeted Mutagenesis Core Facility into pronuclei of NOD mouse embryos. Founder mice carrying the B6.CTLA-4/foxed-Exon 2)-BAC-transgene were crossed to the VAV-Cre deleter strain (termed NOD-Tg-Cre mice) (37). Vav1-Cre transgenic mice were a gift from Dr. D. Kioussis (Division of Molecular Immunology, MRC National Institute for Medical Research, London, U.K.). In some studies, the mice were further bred onto the NOD.CTLA-4 KO or NOD.BDC2.5-CTLA-4 KO strains, which were generated and maintained in our laboratory (7). All mice were backcrossed and maintained on the NOD background. Mice were housed in a specific pathogen-free facility at the University of California San Francisco Transgenic/Targeted Mutagenesis Core Facility into pronuclei of NOD mouse embryos. Founder mice carrying the B6.CTLA-4/foxed-Exon 2)-BAC-transgene were crossed to the VAV-Cre deleter strain (termed NOD-Tg-Cre mice) (37). Vav1-Cre transgenic mice were a gift from Dr. D. Kioussis (Division of Molecular Immunology, MRC National Institute for Medical Research, London, U.K.).

Assessment of diabetes and insulitis

Diabetes development was assessed weekly by testing either urinary or blood glucose levels with Diastix (Bayer) or a Lifescan glucose meter (One Touch II; Lifescan, Roche), respectively. Mice were considered diabetic after two consecutive measurements of >250 mg/dL. Diabetes was also induced in 8-wk-old prediabetic mice by two i.p. injections of cyclophosphamide (200 mg/kg) on days 0 and 7. Diabetes onset was monitored daily for 36 d.

FACS-sorted CD4+/CD62Lhi/CD25 T naive cells from lymph nodes of NOD and NOD-Tg-Cre mice were stimulated with soluble anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of 200 U/ml IL-2. The number of cells indicated in each experiment was adoptively transferred into full NOD recipient mice by retro-orbital injection. Diabetes development was monitored daily for 30 d as described above. NOD.CD28KO recipients (5 wk old) were injected with 5×10^5 primary CD4+/CD62Lhi/CD25 Treg cells, isolated from spleen and lymph nodes of NOD.BDC2.5-transgenic donor mice. Diabetes development was assessed weekly as described above.

Real-time PCR analysis

RNA was isolated from sorted CD4+ T conventional or CD4+/CD62Lhi/CD25 Treg cells with an RNeasy Kit (Qiagen) and reverse transcribed using a Superscript kit (Invitrogen), according to the manufacturers’ instructions. The amounts of li-CTLA-4 and fl-CTLA-4 cDNA were measured using quantitative real-time PCR analysis (GeneAmp 7900; Applied Biosystems) and were normalized to 18S (Eukaryotic 18S rRNA). The TaqMan probe/primer probe for 18S was purchased from Applied Biosystems (ID: 4333760F). Primers and probes for li-CTLA-4 and fl-CTLA-4, according to Gerold et al. (39), were purchased from Integrated DNA Technologies.

Results

Generation of CTLA-4, Exon 2 floxed BAC-transgenic mice and selective expression of li-CTLA-4

CTLA-4 transgenic mice were generated using a BAC construct that contains the entire B6.CTLA-4 locus. This strategy ensures appropriate expression of both the fl- and the li-CTLA-4 isoform by using the gene’s endogenous promoter. The B6.BAC was further engineered to express loxP sequences flanking Exon 2 of the CTLA-4 gene. Exon 2 encodes the extracellular, B7 binding domain of the CTLA-4 molecule, and thus, Cre-mediated deletion of Exon 2 results in expression of only the li isoform from the transgene. First, we introduced the B6.BAC into the full NOD background. Selective expression of li-CTLA-4 from the B6.BAC was achieved by breeding founders to a Vav-Cre deleter strain (Fig. 1A). In some experiments, mice expressing the transgene were backcrossed onto the NOD.BDC2.5-CTLA-4KO background resulting in distinct lines that could be used to examine the role of li-CTLA-4 in normal NOD (NOD-Tg-Cre) and TCR transgenic, CTLA-4-deficient settings (Fig. 1B).

We first evaluated the effects of expressing “B6” levels of the li-CTLA-4 isoform in the li-CTLA-4–deficient NOD background (Fig. 1B, left panel). Real-time PCR analyses were performed to measure and compare expression levels of li- and fl-CTLA-4 in FACS-sorted CD4+ T cells from B6, wild-type (WT) NOD, and NOD-Tg-Cre mice. Consistent with previous observations (28, 34), there were significantly lower levels of li-CTLA-4 mRNA levels in
autoimmune-susceptible NOD mice as compared with B6 mice. In contrast, li-CTLA-4 mRNA levels in NOD-Tg-Cre mice resembled that observed in B6 mice, demonstrating successful expression of li-CTLA-4 from the BAC transgene at physiological expression levels (Fig. 1C, left panel). Moreover, fl-CTLA-4 mRNA levels were equivalent when comparing T cells from B6, NOD, and NOD-Tg-Cre mice, suggesting that the expression of the BAC did not alter fl-CTLA-4 expression (Fig. 1C, right panel). Specific analyses of CTLA-4 intracellular and cell surface expression suggested that NOD-Tg-Cre T cells expressed slightly higher surface levels of the fl-CTLA-4 molecule in the CD4+/CD44+ T effector/memory compartment; however, those changes did not reach statistical significance (Fig. 2A, Supplemental Fig. 1A, left panel).

Expression of increased levels of li-CTLA-4 does not alter the overall T cell numbers or phenotype in prediabetic NOD mice

The autoimmune susceptible strain NOD is genetically deficient in the li-CTLA-4 isoform (28) due to a single nucleotide polymorphism that alters CTLA-4 gene splicing. Increased expression of li-CTLA-4 in NOD-Tg-Cre mice, at levels observed in B6 mice, did not alter the overall immune cell subset distribution and activation state. There were equal protein expression levels of CD25, GITR, and ICOS, suggesting that there were no differences in the activation status of T conventional versus Treg cells (Fig. 2A-C, Supplemental Fig. 1A, right panel). Furthermore, total cellularity of the lymphatic organs, spleen, axillary lymph nodes and pancreatic lymph nodes (pancLN) was unaltered (Fig. 2D). In addition, comparable numbers of total CD4+ T conventional, as well as effector/memory and naive T cells (Supplemental Fig. 1B), were observed when comparing the NOD-Tg-Cre to WT-NOD mice.

NOD mice expressing li-CTLA-4 at B6 levels have reduced insulitis and are protected from cyclophosphamide-induced diabetes

Using our NOD.CTLA-4.BAC-transgenic mouse, which selectively expresses li-CTLA-4 at B6 levels, allowed us to address the biological significance of this CTLA-4 isoform in the context of T1D development in the NOD mouse. T1D development in 8-wk NOD and NOD-Tg-Cre mice was examined following two injections of 200 mg/kg cyclophosphamide at days 0 and 7, which has been shown to synchronize and accelerate disease onset and has been used to study NOD.Idd5.1-congenic mice. We observed a significant reduction in disease frequency with only 42% incidence in NOD-Tg-Cre mice as compared with 88% in WT littermate controls (Fig. 2A).
3A). The level of protection was comparable to the one observed in cyclophosphamide-treated NOD.Idd5.1-congenic mice (40). Next, we compared the degree of insulitis in 20- to 24-wk-old prediabetic NOD and NOD-Tg-Cre mice. The individual islets were scored based on the severity of insulitis (38). The degree of islet infiltration was significantly reduced in NOD-Tg-Cre mice as compared with WT littermates (Fig. 3B). All mice used were normal glycemic. These results confirm other genetic studies, which suggested that the reduced disease observed in NOD.Idd5.1-congenic mice was due to increased li-CTLA-4 expression (34).

Expression of li-CTLA-4 in NOD mice effects T effector cells but not Treg cells

The clinical result described above would be consistent either with changes in the activation state of the conventional T cells or changes in the Treg population. Therefore, we examined the effect of altered li-CTLA-4 expression on individual T cell subsets. Quantitative PCR was performed to measure expression levels of fl- and li-CTLA-4 in Foxp3+ T cells in WT NOD versus NOD-Tg-Cre Treg cells. We identified similar levels of fl-CTLA-4 mRNA but increased li-CTLA-4 expression in Tregs from NOD-Tg-Cre mice compared with WT littermates (Fig. 4A), which reflects the higher mRNA levels of li-CTLA-4 from the B6 allele. These results were similar to what we observed in CD4+ T conventional cells (Fig. 1C). However, there were no detectable differences in the absolute numbers of Foxp3+ T cells or in Foxp3 expression levels on a per cell basis between NOD and NOD-Tg-Cre mice (Fig. 4B). These results were consistent with recent studies suggesting that the intracellular domain of CTLA-4 controls some aspects of Treg function (27). Further analyses of the thymically derived natural versus peripherally developing adaptive Treg subsets in the NOD-Tg-Cre mice were performed based on the expression of the transcription factor Helios (41, 42). The percentage and ratio of natural Treg (Foxp3+/Helios+) versus adaptive Treg (Foxp3+/Helios−) cells were not altered when compared with WT NOD mice (Fig. 4C). Although there were no changes in the overall Treg cell phenotype under steady-state conditions in prediabetic NOD-Tg-Cre mice, we addressed whether increased levels of the li-CTLA-4 protein in Tregs affected their suppressive activity. NOD-Tg-Cre mice were bred with the BDC2.5 TCR transgenic mouse strain. The TCR expressed on the T cells in this mouse recognizes an islet autoantigen, chromogranin A (43). Previous studies have shown that BDC2.5 T effector cells can induce T1D upon adoptive transfer (44), whereas the transfer of BDC2.5 Tregs can block the development of diabetes when transferred into NOD or highly susceptible Treg-deficient, CD28-deficient NOD mice (45–47). Thus, purified Tregs from NOD.BDC2.5 and NOD.BDC2.5-Tg-Cre mice were adoptively transferred into NOD.CD28KO mice at 5 wk of age. As shown in Fig. 4D, the adoptive transfer of 5 × 10⁶ BDC2.5.NOD Tregs delayed disease onset and decreased T1D incidence (66% as compared with 100% in untreated recipients). Similar results were observed using BDC2.5 Tregs derived from the NOD-Tg-Cre mice. Thus, expression of li-CTLA-4 at B6 levels in the full NOD background does not alter Treg cell function in vitro (data not shown) or in vivo.

Attention was, therefore, focused on the T conventional populations to determine what affect higher expression of li-CTLA-4 had on the development and function of T effector cells. As seen in Fig. 4E, there was a marked decrease in the percentage of activated T effector cells in the pancLN and pancreas of NOD-Tg-Cre mice.
In the pancLN, the percentage of activated CD4+/CD44+ T cells was reduced from 15 ± 0.46% in WT to 10.5 ± 1.1% in Tg-Cre mice. Similarly, the percentage of CD4+ T cells infiltrating the pancreas were decreased from 9.6 ± 2% in WT NOD mice to 3.8 ± 0.65% in Tg-Cre mice. Because we observed that re-expression of li-CTLA-4 in NOD mice does not affect the overall phenotype and function of Treg cells, it was likely that altered T effector cell expansion was an intrinsic effect on this T cell subset and not reflective of changes in Treg activity. To directly examine the possibility that li-CTLA-4 negatively affects T cell activation, NOD and NOD-Tg-Cre CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for 3 d, and IFN-γ production was examined by flow cytometry. Under these conditions, there was significantly reduced induction of IFN-γ-producing CD4+ T cells in NOD-Tg-Cre mice as compared with WT littermates (Fig. 4F), suggesting that increased expression of the li-CTLA-4 isoform altered T cell activation because of the increased ability of the cytoplasmic domain of CTLA-4 to alter T cell signal transduction.

Selective expression of the li-CTLA-4 isoform in the fl-CTLA-4-deficient background does not protect from T1D development

Previous studies by our group and others have shown that ligand-independent forms of CTLA-4 can alter the progression of the hyperproliferation observed in the CTLA-4-deficient setting (34, 48). However, those studies did not address the potential importance of expression of the fl-CTLA-4 in the context of Ag-specific T cell responses in the T1D setting. Therefore, we bred the NOD-Tg and NOD-Tg-Cre mice onto the NOD.BDC2.5.CTLA-4KO background, as Mathis and colleagues (11) had previously shown that breeding the CTLA-4 KO to the NOD.BDC2.5-TCR transgenic mouse rescued the systemic autoimmune phenotype observed in straight CTLA-4 KO mice. Moreover, they observed that these mice exhibited accelerated diabetes development between 6 and 8 wk of age (11). This allowed us to distinguish the effects of the li-CTLA-4 isoform in a selective Ag-specific TCR Tg background from effects on the overall disruption of immune homeostasis seen in CTLA-4 KO mice that leads to death in 2–5 wk. Mice on the BDC2.5.CTLA-4KO background expressing both the fl- and li-CTLA-4 isoforms from the B6-BAC in the absence of the VαV-Cre transgene were referred to as BDC-KO-Tg mice. For comparison, BDC2.5.CTLA-4KO mice expressing the VαV-Cre transgene and thus only express li-CTLA-4 from the B6-BAC are referred to as BDC-KO-Tg-Cre mice (Fig. 1B, right panel). First, we compared the level of expression the both CTLA-4 isoforms in CD4+ T cells isolated from NOD.BDC2.5.CTLA-4 WT, KO, KO-Tg, or KO-Tg-Cre mice. As expected, only the li-CTLA-4 isoform was observed in the BDC-KO-Tg-Cre T cells, indicating that the expression of the Cre resulted in a highly efficient recombination of floxed Exon 2 (Fig. 5A). In contrast, similar mRNA and protein levels of fl-CTLA-4 could be detected in BDC-WT and BDC-KO-Tg mice (Fig. 5A, Supplemental Fig. 2A). There was increased expression of li-CTLA-4 in BDC-KO-Tg and BDC-KO-Tg-Cre mice as compared with BDC-WT NOD mice consistent with the inherent genetic deficiency of the li form in this genetic setting. Importantly, the expression of both the fl and the li isoform from the full BAC transgene in BDC-KO-Tg mice completely rescued the mice from increased lymph node cellularity (Fig. 5B) as well as the development of a highly activated T cell phenotype and increase in CD25+Foxp3+ Treg cells detected in BDC.CTLA-4KO mice at 6 wk of age (Fig. 5C). The physiologic expression of only the li-CTLA-4 isoform in BDC-KO-Tg-Cre mice rescued the highly increased lymph node cellularity.
This resulted in a significant but not total reduction of CD4+/CD44+ T effector cells in BDC.CTLA-4KO mice (Fig. 5B, 5C, Supplemental Fig. 2B). Similarly, the percentage and total number of CD4+/Foxp3+ Tregs in BDC-KO-Tg-Cre mice was comparable to those observed in BDC-KO-Tg and WT littermates (Fig. 5C, Supplemental Fig. 2B).

Next, we determined the effect of the different isoforms on diabetes incidence by comparing NOD.BDC2.5.CTLA-4 WT, KO, KO-Tg, and KO-Tg-Cre mice (Fig. 6A). We found that BDC-KO-Tg mice, expressing fl- and li-CTLA-4 from the BAC-transgene, had a lower incidence of T1D than WT NOD.BDC2.5 littermates (Fig. 6A). Although the difference in disease incidence did not reach statistical significance, these results corroborate those described in the conventional NOD-Tg-Cre mice in which the higher li-CTLA-4 expression introduced by the B6 allele significantly ameliorates disease (Fig. 3). The results support a key role of the li-CTLA-4 isoform in controlling T cell autoreactivity independent of TCR specificity. However, when the li-CTLA-4 isoform was examined in the absence of the fl molecule, a different outcome was observed. The NOD.BDC2.5-KO-Tg-Cre mice expressing only the B6-BAC–derived li-CTLA-4 isoform developed diabetes with the same kinetics observed in straight NOD.BDC2.5.CTLA-4KO mice. Thus, despite the partial rescue of the generalized activated T cell phenotype in BDC-KO-Tg-Cre mice, expression of the ligand non-binding CTLA-4 isoform did not confer protection from autoimmunity in the absence of the fl molecule.

Next, we examined whether the requirement of fl-CTLA-4 in conjunction with li-CTLA-4 isoform was essential on T effector and or Treg cells. Adoptive transfer experiments were performed in which NOD.BDC2.5.CTLA-4 WT, NOD.BDC2.5.CTLA-4 KO, and NOD.BDC2.5.CTLA-4 KO-Tg-Cre Tregs were transferred into NOD.CD28KO recipients to compare their relative in vivo suppressive activity. As depicted in Fig. 6B, adoptive transfer of Tregs from NOD.BDC2.5.CTLA-4 WT, NOD.BDC2.5.CTLA-4 KO, or NOD.BDC2.5.CTLA-4 KO-Tg-Cre mice displayed equal suppressive activity, similarly reducing diabetes incidence. Thus, it appears that the absence of the fl-CTLA-4 molecule in BDC-KO-Tg-Cre Tregs did not account for the loss of disease protection in those mice.

Finally, we examined the potential of NOD.BDC2.5.CTLA-4 WT, NOD.BDC2.5.CTLA-4 KO, and NOD.BDC2.5.CTLA-4 KO-Tg-Cre T effector cells to cause diabetes in an adaptive transfer model. One million in vitro-activated T effector cells were transferred into full NOD recipients. The BDC2.5 T effector cells expressing only the li-CTLA-4 isoform induced disease more rapidly as compared with BDC-WT cells expressing both the truncated and fl-CTLA-4 molecules. Moreover, disease kinetics was comparable when trans-
ferring BDC2.5-T effectors expressing only the li-CTLA-4 isoform and T effectors completely deficient for CTLA-4 (Fig. 6C). In addition, the adoptive transfer of 5 \times 10^6 WT-BDC2.5 T effectors caused disease in only 33% of full NODs, whereas the same number of NOD.BDC2.5.CTLA-4 KO or NOD.BDC2.5.CTLA-4 KO-Tg-Cre T effectors induced diabetes in 100 and 75% of recipients, respectively (Supplemental Fig. 3A). Similar results were observed following the adoptive transfer of 1 \times 10^6 naive BDC2.5 T cells in NOD.RAG KO mice (data not shown). The differences in disease incidence (100 versus 75%) upon transfer of 5 \times 10^6 CTLA-4KO versus KO-Tg-Cre T effector cells could be due to a decrease in IFN-\gamma-producing T effector cells in KO-Tg-Cre mice as compared with CTLA-4KO littermates (Supplemental Fig. 3B). Chikuma et al. (48) reported a similar finding of intermediate IFN-\gamma production in T cells overexpressing li-CTLA-4 when compared with WT and CTLA-4KO T cells. Thus, T cells from KO-Tg-Cre mice are more potent in transferring disease as compared with WT T cells but appear to have slightly diminished T effector function when compared with T cells from CTLA-4-deficient animals. It should be noted that we were unable to examine selective expression of li-CTLA-4 in Treg cells only using the Foxp3-Cre-deleter strain (49) because we could not obtain homozygous CTLA-4KO mice carrying the Foxp3-Cre-Tg. It appeared that the insertion site of the Foxp3-Cre-Tg is in close proximity to the endogenous CTLA-4 locus, thus preventing independent heredity/transmission of both alleles. However, taken together, the data indicate that despite the presence of the li-CTLA-4 isoform, the absence of an fl, B7 binding, CTLA-4 molecule results in a more aggressive T effector population and that even low numbers of Ag-specific li-CTLA-4 T effectors can confer disease development. These results are summarized in Table I.

### Table I. Comparative summary of results obtained upon selective expression of li-CTLA-4

<table>
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<td>n.d</td>
<td>Increased compared with WT</td>
<td>Reduced compared with KO</td>
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</table>

n.d., Not determined; +, sufficient for indicated CTLA-4 isoform; −, deficient for indicated CTLA-4 isoform.
NOD-Tg-Cre mice showed greatly reduced IFN-γ production versus extrinsic mechanisms. Thus, we propose that the two CTLA-4 isoforms have distinct and li-CTLA-4 first upregulated upon T cell activation (29). isoforms, with li-CTLA-4 preferentially expressed in naive T cells engagement to control autoimmunity in this Ag-specific model of stimuli, T cells require CTLA-4 ligand, B7-1- and B7-2, engagement in which the biochemical effects known to be mediated by the interaction of li-CTLA-4 with the fl molecule, appropriate levels of li-CTLA-4 are critical to control T cell activation resulting in reduced disease frequency. We speculate that li-CTLA-4 stabilizes surface expression of the fl molecule in the context of T cell activation, thus enhancing ligand engagement and consequently diminishing T effecter function. This hypothesis is consistent with the observation that increased expression of li-CTLA-4 in NOD mice results in a slight increase of surface expression of fl-CTLA-4. In addition, our previous observations that li-CTLA-4 can be detected together with fl-CTLA-4 in the lipid–raft fraction of activated T cells (9, 12) as well as the direct demonstration of heterodimer formation of li-CTLA-4 with the fl molecule (29) supports this hypothesis.

To address the role of li-CTLA-4 in the context of T1D development, in the absence of the fl molecule, we crossed our BAC-transgenic mice onto the NOD.BDC2.5.CTLA-4KO background. Mathis and colleagues (11) demonstrated that CTLA-4 KO mice on the BDC2.5-transgenic background are protected from CTLA-4 KO disease, namely lymphocyte hyperproliferation and multiorgan infiltration, but develop accelerated T1D by the age of 6–8 wk. We observed that expression of only the li-CTLA-4 isoform in the NOD.BDC2.5.CTLA-4 KO background significantly reduced the number of activated effector/memory T cells and almost completely rescues the highly increased lymph node cellularity observed in CTLA-4 KO littermates. These results point to an important intrinsic function of CTLA-4 independent of B7 engagement. However, selective expression of li-CTLA-4 failed to rescue T1D development observed in CTLA-4 KO mice. We speculate that the partial rescue of the highly activated T cell phenotype reflects an important role of li-CTLA-4 in regulating tonic TCR signaling in which the biochemical effects known to be mediated by the intracellular portion of the molecule raise the activation threshold of naive T cells. However, following acute antigenic stimuli, T cells require CTLA-4 ligand, B7-1- and B7-2, engagement to control autoimmunity in this Ag-specific model of T1D. This is likely considering the differential expression of both isoforms, with li-CTLA-4 preferentially expressed in naive T cells and fl-CTLA-4 first upregulated upon T cell activation (29). Thus, we propose that the two CTLA-4 isoforms have distinct functions in different T cell subsets and that CTLA-4 regulates T cell homeostasis, immune activation, and tolerance by distinct intrinsic versus extrinsic mechanisms.

The fact that expression of only the li form of CTLA-4 results in a significant decrease of activated T cells in three different transgenic mouse models (34, 48) compared with a CTLA-4KO background (7, 8), we hypothesized that this phenotype might be a consequence of improved Treg function by li-CTLA-4. To directly evaluate the relative role of fl-versus li-CTLA-4 in Treg function, we used an in vivo adaptive transfer model of T1D and identified that BDC2.5-transgenic li-CTLA-4 Tregs confer disease protection comparable to WT Tregs. Interestingly, CTLA-4 KO Tregs also suppressed diabetes development in this setting. These results might be surprising given the numerous reports linking CTLA-4 and especially extrinsic CTLA-4 signaling with Treg-suppressive function (50). In particular, the recent study by Sakaguchi and colleagues (51) concludes a Treg-specific role of CTLA-4, as mice lacking CTLA-4 only in Tregs still develop lymphoproliferative disease. Moreover, recent studies by Singer and colleagues (27) demonstrated the requirement of extracellular CTLA-4 on Treg cells to control T effector functions in a model of inflammatory bowel disease. However, the results reported in this paper are consistent with previous studies from our laboratory demonstrating that CTLA-4 is dispensable on Treg function in animal models of T1D (Ref. 52; J.A. Bluestone and Q. Tang, unpublished observations). The reasons for the differential role of CTLA-4 in different autoimmune and hyperproliferative models remains unclear but may reflect the multitude of documented suppressive mechanisms mediated by this specialized T cells subset, including the production of multiple suppressive factors such as TGF-β, IL-10, and IL-35 (52-54). In this regard, we previously demonstrated that CTLA-4 KO Tregs express high levels of TGF-β that is involved in Treg-suppressive function in a model of T1D (52). Thus, it is likely that Treg cells selectively expressing li-CTLA-4 suppress through these bystander mechanisms. Nevertheless, despite normal Treg-suppressive activity, NOD.BDC2.5.CTLA-4 KO-Tg-Cre mice expressing only the li-CTLA-4 isoform develop diabetes with kinetics comparable to NOD.BDC2.5.CTLA-4 KO mice. This suggests a major role of CTLA-4 on effector T cells in this setting. Performing adoptive transfer experiments, we observed that BDC2.5-transgenic T effector cells expressing only li-CTLA-4 were more potent in transferring disease in full NOD recipients compared with WT cells. Disease kinetics were identical with those observed when transferring CTLA-4 KO cells. Notably, low numbers of Ag-specific T effector cells selectively expressing li-CTLA-4 induced T1D development in 75% of recipients compared with only 33% when transferring equal numbers of WT cells. These results might also provide an explanation for the finding that NOD.BDC2.5.KO-Tg-Cre mice are not protected from diabetes, despite a significant reduction of T effectors in those animals. Thus, we propose that the ectodomain of CTLA-4 and therefore ligand binding is particularly crucial to control T effector function. This is in line with a recent study demonstrating that selective blockade of extrinsic CTLA-4 on T effector cells increases tumor immunity (55). Overall, our results demonstrate the biological significance of li-CTLA-4 in T1D development but also suggest that li-CTLA-4 function in the context of autoimmunity strictly depends on the presence of the fl molecule.

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Disclosures
The authors have no financial conflicts of interest.
References


Suppl. Figure 1:

A  

T conventional  

T regulatory

B  

CD4+  

CD44+  

CD62L+
Suppl. Figure 2:

(A) Flow cytometry data showing the percentage of Max for FI-CTLA-4 in different genotypes: WT, KO, KO-Tg, and KO-Tg-Cre. The x-axis represents the log scale of FI-CTLA-4, and the y-axis shows the percentage of Max. The legend indicates the different genotypes.

(B) Absolute numbers of CD4+ cells and FoxP3+ cells for different genotypes: BDC-WT, BDC-KO, BDC-KO-Tg, and BDC-KO-Tg-Cre. The graphs display the data with statistical significance indicated by asterisks (*, **, ***).
Suppl. Figure 3:

A. % diabetes incidence over time for different groups of Teffs.

B. IFN-γ fold change for different groups.
**Suppl. Figure 1:** (A) Quantification of CTLA-4, CD25, GITR and ICOS protein expression levels in CD4+ T conventional and FoxP3+ T regulatory cells from NOD and NOD-Tg-Cre mice. (B) Absolute numbers of conventional T cells (CD4+), activated T effectors (CD44+) and resting naïve T cells (CD62L hi) determined by flow cytometry and displayed as percentage of total pancreatic LN cells in NOD and NOD-Tg-Cre mice.

**Suppl. Figure 2:** (A) Protein expression levels of full-length CTLA-4 (FL-CTLA-4) in CD4+ LN cells from NOD.BDC2.5.CTLA-4 WT, KO, KO-Tg or KO-Tg-Cre mice. The anti-CTLA-4 antibody used in the flow cytometry analysis selectively recognizes the extracellular domain of the receptor. (B) Absolute numbers of conventional T cells (CD4+), activated T effectors (CD44+) and regulatory T cells (FoxP3+) in lymph node from NOD.BDC2.5.CTLA-4 WT, KO, KO-Tg or KO-Tg-Cre mice.

**Suppl. Figure 3:** (A) Diabetes frequency in full NOD recipients after adoptive transfer of 5x10^5 in vitro activated BDC2.5-T effector cells from CTLA-4 WT, KO or KO-Tg-Cre mice, respectively. (B) Fold changes of IFN-γ producing CD4+ T cells from sorted and in vitro activated CD4+CD62L hi T naïve cells of NOD.BDC2.5.CTLA-4 WT, KO and KO-Tg-Cre mice.