Inhibition of T cell activation and autoimmune diabetes using a B cell surface–linked CTLA-4 agonist

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CTL-associated antigen 4 (CTLA-4) engagement negatively regulates T cell activation and function and promotes immune tolerance. However, it has been difficult to explore the biology of selective engagement of CTLA-4 in vivo because CTLA-4 shares its ligands, B7-1 and B7-2, with CD28. To address this issue, we developed a Tg mouse expressing a single-chain, membrane-bound anti–CTLA-4 Ab (scFv) on B cells. B and T cells developed normally and exhibited normal phenotype in the steady state and after activation in these mice. However, B cells from scFv Tg mice (scFvCTLA4+) prevented T cell proliferation and cytokine production in mixed lymphocyte reactions. Additionally, mice treated with scFvCTLA4+ B cells had decreased T cell–dependent B cell Ab production and class switching in vivo after antigen challenge. Furthermore, expression of this CTLA-4 agonist protected NOD mice from spontaneous autoimmune diabetes. Finally, this disease prevention occurred in Treg-deficient NOD.B7-1/B7-2 double-knockout mice, suggesting that the effect of the CTLA-4 agonist directly attenuates autoreactive T cell activation, not Treg activation. Together, results from this study demonstrate that selective ligation of CTLA-4 attenuates in vivo T cell responses, prevents development of autoimmunity, and represents a novel immunotherapeutic approach for the induction and maintenance of peripheral tolerance.

Introduction

CTL-associated antigen 4 (CTLA-4, also known as CD152) has been well established as a negative regulator of T cell function (1–3). CTLA-4 ligation antagonizes early T cell activation, leading to decreased IL-2 production, inhibition of cell cycle progression, decreased induction of cyclins by anti-CD3, and modulation of TCR signaling (2, 4–6). Mice deficient for CTLA-4 develop lymphoproliferative disease and die within 4 weeks of birth (7, 8) adding further evidence for the critical role of CTLA-4 inhibition of T cell responses. More recently, CTLA-4 has been implicated in the function of Tregs, which suppress effector T cell activation and function (9, 10). In fact, it is clear that CTLA-4 plays a critical role in both the induction and maintenance of immune tolerance (11, 12). Thus, many efforts have focused on developing therapeutic approaches to selectively engage this pathway to treat autoimmunity and organ transplant rejection. Unfortunately, these attempts have been largely unsuccessful due to the complex biology of CTLA-4 (13). Early studies using Abs to target the CTLA-4 ligands B7-1 (also known as CD80) and B7-2 (also known as CD86) for immune regulation have been unreliable, as these ligands are shared with the positive costimulatory molecule CD28. Thus, rather than engaging CTLA-4 to turn off immunity, the ligands promote CD28/B7 interactions, resulting in enhanced T cell activation, expansion, and differentiation. Second, in vivo administration of anti–CTLA-4 Abs routinely acts as an antagonist of CTLA-4 function. For instance, treatment of autoimmune-prone mice with anti–CTLA-4 results in the exacerbation of diseases, including experimental autoimmune encephalomyelitis and autoimmune diabetes (14, 15). Thus, a new approach to directly engage the CTLA-4 molecule with an agonist signal is essential for treating autoimmunity.

CTLA-4 signaling requires cross-linking in conjunction with T cell receptor engagement. This requirement is a direct consequence of the fact that CTLA-4 functions by directly influencing TCR-mediated signal transduction by altering TCR-ζ chain phosphorylation and immune synapse formation (6, 16, 17). Thus, any effective negative signal following CTLA-4 engagement depends on the molecule being engaged, both physically and temporally, together with the TCR signal from the same APC (6). This most likely explains why in vivo treatment of mice with anti–CTLA-4 Abs acts as an antagonist, since even Fc receptor–mediated cross-linking would not likely occur with a cell bearing the relevant antigen. We hypothesized that effective delivery of a CTLA-4 agonist would depend on presentation of a specific CTLA-4 ligand (i.e., anti–CTLA-4 Ab) on a relevant APC-bearing cognate antigen. Thus, we developed a single-chain, membrane-bound anti–CTLA-4 Ab (scFv) that can be expressed directly on APCs. In previous studies, we demonstrated that this reagent was effective in vitro in blocking T cell activation and, as predicted, was effective when presented in cis, on the same APC, during TCR signaling (18). In this study, we have addressed the in vivo potential of such a reagent in an autoimmune setting. A construct was generated and expressed selectively on antigen-presenting B cells, which have been shown to be essential in secondary immunity and autoimmune diabetes in the NOD mouse strain (19). The model also takes advantage of the...
motility of B cells that traffic to sites of antigen priming and participate in autoimmune reactions at sites of inflammation. Using this Tg mouse model, we demonstrate that cell surface–bound anti–CTLA-4 does not alter normal B or T cell development or function but selectively inhibits antigen-specific T cell function in vivo and the development of autoimmune diabetes.

Results

Generation of membrane-bound anti–CTLA-4 Tg+ mice. The anti–CTLA-4 (clone 4F10) single-chain, membrane-bound (4F10scFv) construct has been well characterized in vitro and shown to have profound inhibitory effects on T cell function (18, 20). These results prompted us to develop a Tg mouse model to test this novel construct in vivo. We chose to express the molecule on B cells since previous studies showed that the scFv CTLA-4 was most effective when coexpressed on APCs, and B cells would be an effective cell population to selectively test antigen-specific responses. Thus, the IgM heavy-chain promoter and enhancer (PmEm) was used to drive the expression of single-chain, anti–CTLA-4 (7M−4F10scFv) (21). In order to ensure that there would be no reverse signaling to the scFv-expressing cells, the construct was created using the transmembrane domain from B7-1, which lacked the C terminal intracytoplasmic tail (18, 20). The PmEm 7M-4F10scFv B7-1 construct was microinjected into day 1 embryos isolated from FVB/n mice. B cell–specific 7M-4F10scFv B7-1 Tg+ mice (hereafter referred to as scαCTLA-4) developed normally according to Mendelian fre-

Figure 1

Tg+ scαCTLA-4 expression. Flow cytometric analysis was performed on splenocytes from scαCTLA-4 Tg+ mice and littermate controls using soluble murine fusion protein mCTLA-4 Ig and anti-B220. (A) Upper panels demonstrate scαCTLA-4 on naive Tg+ B cells and activated Tg+ B cells following anti-IgM stimulation (48 hours, 1 μg/ml) compared with FVB littermate negative controls (lower panels). All plots were gated on B220+ B cells. (B) Flow cytometric analysis was performed on thymocytes from scαCTLA-4 Tg+ mice and littermate controls as described above. Flow plots illustrate scαCTLA-4 expression in DP CD4+CD8+ thymocytes from scαCTLA-4 Tg+ mice, but not DP CD4+CD8+ thymocytes from FVB or SP CD4+ and SP CD8+ from scαCTLA-4 Tg+ or FVB Tg− controls. Results shown are representative of 3 independent experiments with ≥ 3 mice per group.

Figure 2

Characterization of T cells and B cells in scαCTLA-4 Tg+ mice. Flow cytometric analysis was performed to determine the percentage of CD4+ T cells, CD8+ T cells, and B220+ B cells in the spleen, peripheral lymph nodes, and thymus from FVB and FVB.scαCTLA-4 Tg+ mice. The number in each histogram quadrant represents the percentage of total cells. Results shown are representative of 3 independent experiments with ≥ 3 mice per group.
The frequencies and without gross defects. Tg scCTLA-4 expression was identified by flow cytometry using murine CTLA-4 Ig. scCTLA-4 expression was found on B cells isolated from the spleen and peripheral lymph nodes. Approximately 55% of the B220+ B cells isolated from spleen of Tg mice were CTLA-4 Ig positive, with a range of 30%–60% (Figure 1A). This staining specificity was confirmed using human CTLA-4 Ig, which did not bind (data not shown). Stimulation of B cells with anti-IgM or LPS significantly increased the expression of scCTLA-4 Tg* to more than 80% of the B220+ B cells (Figure 1A). scCTLA-4 expression was also detected on CD4+CD8+ double-positive (DP) thymocytes but not on single-positive (SP) CD4+ or CD8+ thymocytes (Figure 1B), peripheral CD4+ and CD8+ T cells, macrophages, or DCs (data not shown). It is also important to note that activated CD4+ and CD8+ T cells did not express scCTLA-4 following anti-CD3 and anti-CD28 activation (data not shown).

T and B cells develop normally in scCTLA-4 Tg* mice. Despite scCTLA-4 expression on DP thymocytes, T cell development appeared normal. This was evident in the thymus, with 70% CD4+CD8+ DP, 9% CD4+ SP, and 3% CD8+ SP thymocytes for both scCTLA-4 Tg* and littermate controls (Figure 2). Moreover, we did not find any changes in the peripheral T cells from lymphocytes nor significant changes in the total spleen cellularity, based on total numbers or composition. Approximately 55% B220+ B cells, 30% CD4+ T cells, and 15% CD8+ T cells were observed in both groups (Figure 2). Additionally, we did not find any differences in marginal zone, follicular B cells, or transitional B cells in Tg* mice compared with controls (data not shown). CD4+ T cells constituted approximately 60% of the T cell composition of peripheral lymph nodes, with 25% being CD8+ T cells (Figure 2). These data indicated that although scCTLA-4 expression was detected in the DP thymocytes, T and B cell development was normal.

scCTLA-4 Tg* B and T cell function. The functional response of splenocytes (Figure 3, A and B) or purified B cells (data not shown) from scCTLA-4 Tg* mice and littermate controls was compared using anti-IgM or LPS as mitogens. B cell proliferation, Ab production, IgM, MHC class II, CD40, B7-1, and B7-2 expression were measured. The expression of the Tg did not alter B cell responsiveness to BCR or innate stimuli (Figure 3, A and B). In addition, the surface expression of CD40, B7-1, B7-2, and MHC class II on naive and activated Tg* and Tg* B cells was comparable at 24, 48, and 72 hours (Figure 3D and data not shown).

We next assessed the functional potential of the peripheral T cell compartment. Purified T cells from spleen and peripheral lymph nodes were stimulated with plate-bound anti-CD3. Peripheral T cells isolated from scCTLA-4 Tg* mice proliferated in a dose-dependent response to TCR engagement in a manner similar to control FVB T cells (Figure 3C). CD69 upregulation and CD62L
downmodulation were also normal upon activation in Tg− T and B cells (data not shown). Collectively, these data indicate that the Tg expression of sccCTLA-4 on B cells or DP thymic T cells did not significantly alter the development or function of peripheral B and T cells to polyclonal stimuli, including LPS, anti-IgM, or anti-CD3 and anti-CD28.

sccCTLA-4 Tg+ B cells inhibit T cell proliferation and cytokine production. Next, we examined whether the expression of sccCTLA-4 on allogeneic B cells would alter T cell activation in vitro in a mixed lymphocyte reaction (MLR). Responder C57BL/6 T cells were stimulated with MHC-mismatched purified CD19+ B cells from FVB sccCTLA-4 Tg+ mice or littermate controls. T cell proliferation in response to sccCTLA-4 Tg− allogeneic MHC was significantly lower (>50% reduction, P < 0.0002 at 1:5 responder/stimulator ratio or higher) compared with control B cells (Figure 4A). Total splenocytes from FVB and FVB.sccCTLA-4 were not as effective (30% reduction) at inhibiting T cell priming as purified B cells (50%), suggesting endogenous DC activated the alloreactive T cells (data not shown). These data also suggest that sccCTLA-4 function did not inhibit in trans although the 30% reduction in proliferation might suggest either a trans effect or, more likely, engagement of the activated T cells with antigen-bearing non-Tg B cells or DCs. In this regard, stimulation with purified sccCTLA-4 Tg+ B cells inhibited effector cytokine production in the allogeneic MLR. IFN-γ production was suppressed by 83% (P < 0.006) and IL-2 production by 68% (P < 0.02) (Figure 4, C and D). IL-4 and IL-10 were undetectable, indicating there was not a switch to a Th2-type response (data not shown). The reduced response was dependent on CTLA-4 expression on responding T cells, as C57BL/6 T cells from CTLA-4 KO mice responded equally to sccCTLA-4 Tg+ and Tg− B cell stimulations (Figure 4B). Thus, our results demonstrate that anti-CTLA-4 single-chain Ab expressed on APCs act as a CTLA-4-specific agonist to block responses of cognate T cells.

sccCTLA-4 inhibits T cell–dependent B cell Ab production in vivo. We next determined the effects of sccCTLA-4 on immunological responses to hapten-carrier complexes in vivo. WT FVB mice received dinitrophenyl-OVA–pulsed (DNP-OVA–pulsed) B cells from either sccCTLA-4 Tg+ mice or FVB controls (priming event). Seven days following initial priming, recipient mice received a boost of OVA antigen in CFA subcutaneously. DNP-specific Ab levels in sera were determined by using antigen-specific ELISA to determine levels of IgG1, IgG2a, and total Ig (days 0, 7, and 14). Mice immunized with antigen-pulsed B cells from the sccCTLA-4 Tg+ mice showed significantly decreased DNP-specific Abs (Figure 5A, P < 0.0009). There was a particular suppression of class switching to IgG2a, suggesting coengagement of CTLA-4 during antigen exposure led to a profound suppression of Th1-dependent Ab production. We did not detect significant differences in levels of IgG1 anti-DNP Abs, indicating that sccCTLA-4 did not cause a switch to a Th2 Ab response (Figure 5B) consistent with the in vitro cytokine responses. Furthermore, we did not detect a significant change in total Ig levels, indicating that the effects of sccCTLA-4 were limited to antigen-specific responses (Figure 5C). These results demonstrate the important role CTLA-4 has in regulating T cell activity using a novel Tg approach that directly engages CTLA-4 on T cells in vivo influencing both T cell and T cell–dependent B cell responses.

Effects of CTLA-4 in 2 models of autoimmune diabetes. The sccCTLA-4 Tg+ mice were crossed onto the autoimmune diabetes-prone NOD...
mouse model to determine the effect of sccCTLA-4 Tg+ B cells inhibit T cell–dependent B cell IgG2a Ab production in vivo. sccCTLA-4 Tg and FVB littermate control B cells were activated in the presence of LPS (1 μg/ml) for 72 hours, loaded with DNP-OVA, and transferred to naive FVB recipients. Seven days following transfer of cells, recipient animals were boosted with 100 μg DNP-OVA in CFA injected subcutaneously. Fourteen days following B cell transfer, anti-DNP Ab production was measured using DNP-KLH–specific ELISA. Shown in A, anti-DNP serum IgG2a from 4 individual WT or sccCTLA-4 Tg+–treated mice. (B) Anti-DNP serum IgG1 from 4 individual WT or scctCTLA-4 Tg+–treated mice. (C) Total Ig from 10 individual WT or scctCTLA-4 Tg+–treated mice. The mean for each group is shown with a horizontal line. Results shown are representative of at least 2 independent experiments.

Figure 5
sccCTLA-4 Tg+ B cells inhibit T cell–dependent B cell IgG2a Ab production in vivo. sccCTLA-4 Tg and FVB littermate control B cells were activated in the presence of LPS (1 μg/ml) for 72 hours, loaded with DNP-OVA, and transferred to naive FVB recipients. Seven days following transfer of cells, recipient animals were boosted with 100 μg DNP-OVA in CFA injected subcutaneously. Fourteen days following B cell transfer, anti-DNP Ab production was measured using DNP-KLH–specific ELISA. Shown in A, anti-DNP serum IgG2a from 4 individual WT or sccCTLA-4 Tg+–treated mice. (B) Anti-DNP serum IgG1 from 4 individual WT or scctCTLA-4 Tg+–treated mice. (C) Total Ig from 10 individual WT or scctCTLA-4 Tg+–treated mice. The mean for each group is shown with a horizontal line. Results shown are representative of at least 2 independent experiments.

Figure 6
NOD.scctCTLA-4 Tg+ mice are protected from spontaneous diabetes. Female NOD.scctCTLA-4 Tg+ mice and NOD littermate controls were monitored for the development of spontaneous autoimmune diabetes. Diabetes incidence is shown here for NOD (n = 17) (filled circles) and NOD.scctCTLA-4 Tg+ (n = 15) (open circles). NOD diabetes incidence reached 70% (12 of 17) by 17 weeks. In contrast, scctCTLA-4 Tg+ mice that were protected from diabetes also had significantly less insulitis (Figure 7B). Although Tregs have been shown to be deficient in NOD.B7 DKO mice (26), it remained possible that the engagement of CTLA-4 by the agonist scctCTLA-4 restored Tregs in the NOD.B7 DKO.scctCTLA-4 Tg+ mice. We addressed this possibility by FACS analysis and determined that Tregs were not restored in NOD.B7 DKO.scctCTLA-4 Tg+ mice. Both B7 DKO groups had 0.3% CD4+CD25hiCD62L+ Tregs in WT NOD mice (Figure 7C). Recent studies suggest that the putative transcription factor FoxP3 is the best marker of mouse Tregs. Therefore, we repeated the analysis of B7 DKO mice, utilizing intracellular stain-
Figure 7

sccCTLA-4 Tg+ NOD.B7 DKO mice are protected from accelerated diabetes. Female NOD.B7 DKO and NOD.B7 DKO.sccCTLA-4 Tg+ mice were monitored for the development of spontaneous disease. (A) Diabetes incidence is shown here for NOD.B7 DKO (n = 12) (open circles) and NOD.B7 DKO.sccCTLA-4 Tg+ mice (n = 20) (filled circles). NOD.B7 DKO diabetes incidence reached 100% while diabetes incidence in NOD.B7 DKO.sccCTLA-4 Tg+ mice was significantly reduced to 60% by 30 weeks of age (P < 0.003). (B) NOD.B7 DKO.sccCTLA-4 Tg+ mice have decreased insulitis at 14 weeks of age. Pancreas from 14-week-old NOD.B7 DKO and sccCTLA-4 Tg+ NOD.B7 DKO mice were stained with H&E to determine clinical severity of insulitis. Pancreatic islets were scored for the presence of mononuclear infiltration: 0, no infiltrate; 1, peri-insulitis present; 2, 25–50% of the islet contained infiltrate; 3, more than 50% of the islet was infiltrated. Average percentages shown were determined from at least 100 islets from at least 3 mice per group. (C) Flow cytometric analysis was performed to determine the percentage of CD4+CD25+CD62L+ and (D) CD4+CD25-FoxP3+ Tregs in the lymphoid tissue of NOD.B7 DKO, NOD.B7 DKO.sccCTLA-4 Tg+, and NOD mice. The mean ± SD from at least 3 mice per group is shown. Results shown are representative of 3 independent experiments.

Next, we determined whether sccCTLA-4 similarly affects the ability of autoreactive BDC2.5 CD4+ T cells to differentiate and produce Th1 effector cytokines such as IFN-γ, which is known to be involved in disease pathogenesis (28). For this study, we utilized an IFN-γ reporter mouse in which the expression of yellow fluorescent protein (YFP) is under the control of the IFN-γ promoter (29). These targeted reporter mice were designated Yeti, for yellow-enhanced transcript for IFN-γ. Direct assessment of in vivo differentiation and IFN-γ production can thus be measured by flow cytometry ex vivo without the requirement of in vitro restimulation. We backcrossed these mice 12 generations to NOD and subsequently bred to NOD.BDC2.5.Thy1.1 TCR Tg+ of the autoreactive BDC2.5 cells went into cycle in the pancreatic LN from NOD.B7 DKO recipient mice compared with only 22.1% (28.8 ± 10.7 %) in NOD.B7 DKO.sccCTLA-4 Tg+ recipients (Figure 8A). Moreover, BDC2.5 CD4+ T cells divided only 3 generations in NOD.B7 DKO.sccCTLA-4 hosts in contrast to 6 to 8 generations in the NOD.B7 DKO recipients (Figure 8A). There was no proliferation observed in antigen-deficient inguinal LN in either NOD.B7 DKO or NOD.B7 DKO.sccCTLA-4 Tg+ mice (Figure 8A). These results indicate that B cells bearing surface-bound anti–CTLA-4 suppress in vivo priming of autoreactive T cells in the absence of Tregs.

Next, we determined whether sccCTLA-4 similarly affects the ability of autoreactive BDC2.5 CD4+ T cells to differentiate and produce Th1 effector cytokines such as IFN-γ, which is known to be involved in disease pathogenesis (28). For this study, we utilized an IFN-γ reporter mouse in which the expression of yellow fluorescent protein (YFP) is under the control of the IFN-γ promoter (29). These targeted reporter mice were designated Yeti, for yellow-enhanced transcript for IFN-γ. Direct assessment of in vivo differentiation and IFN-γ production can thus be measured by flow cytometry ex vivo without the requirement of in vitro restimulation. We backcrossed these mice 12 generations to NOD and subsequently bred to NOD.BDC2.5.Thy1.1 TCR Tg+
mice to derive NOD.BDC2.5.Thy1.1.Yeti mice. The CD4+ T cells from BDC2.5.Thy1.1.Yeti mice were adoptively transferred to prediabetic NOD.B7 DKO or NOD.B7 DKO.sccCTLA-4+ recipients, and the number of YFP-expressing T cells in the pancreatic and inguinal LN were determined by FACS. It is important to note that freshly isolated CD4+ T cells from NOD.BDC2.5.Thy1.1.Yeti mice did not express YFP (data not shown). sccCTLA-4+ Tg+ B cells blocked the IFN-γ production in the pancreatic LN 0.47% ± 0.06% compared with NOD.B7 DKO recipients with 10.3% ± 3% IFN-γ YFP+ positive cells (Figure 8B). CD62L expression was also measured as a parameter of in vivo priming of CD4+ B DC2.5.Thy1.1.Yeti gated cells from pancreatic and inguinal LN. IFN-γ–positive cells from the NOD.B7 DKO recipients downmodulated CD62L further, indicating in vivo activation in the absence of CTLA-4 signal. B DC2.5 cells from NOD.B7 DKO.sccCTLA-4+ recipient mice did not express YFP, and only 35.8% (34.7% ± 1.4%) of the CD4+ cells from pancreatic LN downmodulated CD62L compared with 60.9% (59.9% ± 2.3%) in controls (Figure 8B). The vast majority of CD4+ B DC2.5.Thy1.1 cells (~75%) from the inguinal LN in both groups did not express IFN-γ or downmodulate CD62L, indicating a lack of T cell priming as predicted (Figure 8B). Taken together, the results demonstrate that B cells expressing sccCTLA-4 can efficiently suppress proliferation and differentiation of autoreactive T cells in the draining LN in vivo (30).

Discussion

Although CTLA-4 engagement by B7 has been demonstrated to downregulate T cell responses in several systems, lack of appropriate reagents has prevented direct ligation in vivo to inhibit T cell function in autoimmune diseases. Rather, the majority of studies have utilized CTLA-4 antagonists or ectopic expression of B7 (which stimulate both CD28 and CTLA-4) to implicate the role of CTLA-4 in vivo (refs 14, 15 and reviewed in refs. 13, 31). In the studies presented here, we have generated a model to examine the effect of selective engagement of CTLA-4 during in vivo immune responses. This was accomplished by expressing a single-chain, membrane-bound scFv anti–CTLA-4 selectively on B cells. Our results demonstrate that sccCTLA-4 Tg+ expression did not affect T or B cell development but profoundly inhibited T cell responses to antigens presented by sccCTLA-4 Tg+ B cells in vitro and in vivo. Moreover, we show that selective engagement of CTLA-4 conferred significant protection against disease in 2 models of autoimmune diabetes, even in the absence of Tregs. The mechanism for this regulation was determined to be the prevention of autoreactive T cell expansion and development of effector function.

CTLA-4 has previously been demonstrated to regulate peripheral tolerance in several experimental systems (11, 12, 32). This regulation is at the level of T cell expansion and differentiation. In particular, we have previously shown that selective engagement of CTLA-4 using membrane-bound scFv anti–CTLA-4 inhibits antigen-specific T cell responses in vitro (18, 20). We have also shown that sccCTLA-4 can regulate CD8+ T cell–mediated tumor clearance in an in vivo tumor model (33). CD8+ T cells isolated from animals bearing sccCTLA-4 tumor cells were hyporesponsive ex vivo upon antigen rechallenge, indicating that CTLA-4 engagement resulted in tolerance (33). In this study, we have extended our previous findings to show that sccCTLA-4 expression on B cells inhibits CD4+ autoimmune responses in vivo. Two separate experimental models of autoimmune diabetes were used to address the efficacy of membrane-bound anti–CTLA-4 for disease prevention. We demonstrate that selective engagement of CTLA-4 conferred significant protection against disease in spontaneous NOD diabetes and even in the more aggressive autoimmune NOD.B7-1/B7-2 KO (B7 DKO) model. This CTLA-4 control may function at several
levels, including T cell activation/proliferation, Th1/Th2 differentiation, and cell trafficking. Our data suggest that scctCTLA-4–mediated inhibition is at the level of CD4+ T cell activation/differentiation. We found a significant decrease in the percentage of autoreactive BDC2.5 CD4+ T cells proliferating in the NOD.B7 DKO.scctCTLA-4 Tg+ in pancreatic LN compared with controls, indicating that CTLA-4 engagement had a direct effect on antigen-specific T cell activation and expansion. CD8+ NRP-V7+ tetramer–positive cells were examined in the NOD.scctCTLA-4 Tg+ mice, as these T cells have been shown to be increased during the progression of diabetes in NOD mice (34). There were no statistical differences in the number of CD8+ NRP-V7+ antigen-specific cells in the NOD.scctCTLA-4 Tg+ mice as compared with NOD mice (data not shown). We further demonstrated scctCTLA-4 significantly limited the differentiation of diabetogenic T cells toward Th1 IFN-γ–producing cells. NOD.B7 DKO.scctCTLA-4 Tg+ mice had significant reduction of IFN-γ/YFP+ cells (0.5%) compared with controls (13.3%) in islet-draining pancreatic LN. These data indicate that CTLA-4 engagement significantly inhibits both the proliferation and Th1 differentiation in an antigen-specific response. The protection from disease in this setting appears to be a result of direct inhibition of effector T cell activation/differentiation rather than regulation, as the Treg deficiency observed in NOD.B7 DKO mice was not restored by the expression of the scctCTLA-4 Tg. It is interesting to note that reduced disease incidence correlated with decreased tissue infiltration (Figure 7B). This is not surprising unless you consider the fact that nearly all female NOD mice develop insulitis by 3 to 4 weeks of age (35). This lack of pancreatic infiltration indicates that CTLA-4 may function to prevent the trafficking and accumulation of inflammatory infiltrates, likely at the level of LN priming. It is important to note that the presence of the scctCTLA-4 Tg did not render these mice immunocompromised, as NOD.scctCTLA-4 Tg+ mice cleared cytomegalovirus in a manner similar to that observed in NOD mice. In these experiments, NOD.RAG KO mice were used to demonstrate that immunocompromised animals were unable to clear this viral infection (data not shown). Therefore, the scctCTLA-4–mediated disease inhibition and decreased insulitis in the absence of Tregs support the interpretation that CTLA-4 ligation functioned directly on the autoreactive T cells. These data are consistent with previous findings that CTLA-4 engagement results in a tolerant state, and only after blocking CTLA-4 do tissue infiltration and autoimmunity occur.

scctCTLA-4 Tg+ B cells prevent destructive insulitis and significantly affect NOD and NOD.B7 DKO mice from developing diabetes, suggesting B cells play a pivotal role in disease progression. B cells have been shown to be critical for the development of autoimmune diabetes in NOD mice, as B cell–deficient mice are protected (22, 23). Interestingly, B cell–deficient mice develop some insulitis, suggesting that diabetogenic responses can be initiated in the absence of B cells, likely via DCs. However, diabetes fails to progress in these mice, demonstrating a critical role of B cells in the amplification of autoimmune responses and disease progression (22). Wong et al. recently demonstrated that B cell antigen presentation, not Ig production, is essential for development of insulitis and diabetes (23). In fact, B cells may contribute to disease pathogenesis by further activating T cells in the pancreas or by enhancing epitope spreading where islet Ab–producing B cells selectively process apoptotic β cells to present new islet epitopes. Falcone et al. demonstrated a critical role for B cell antigen presentation and development of spontaneous responses to self antigens during the progression of autoimmune diabetes (19).

Thus, it is possible that in our model, scctCTLA-4 Tg+ expression controlled diabetes by preventing B cell–dependent amplification of the anti-islet responses.

scctCTLA-4 significantly delayed disease onset and development of spontaneous diabetes in NOD mice. Although there was not complete protection, there is a significant consequence of engaging CTLA-4 during development of autoimmune diabetes. There are a number of possibilities as to why B cell expression of anti–CTLA-4 was not sufficient for complete disease protection. First, autoreactive T cells may interact with several APCs during diabetes initiation. Tg B cell expression of anti–CTLA-4 may raise the threshold for T cell activation. However, it is possible that some autoreactive T cells escape scctCTLA-4 regulation and encounter islet antigens presented by DCs or macrophage cells in the absence of CTLA-4. This possibility is supported by an in vitro MLR assay where total splenocytes were used to stimulate MHC mismatched T cells. In this assay, the presence of endogenous Tg nonbearing APCs partially restored T cell proliferation. These data further support the notion that the anti–CTLA-4 is working directly on T effectors and not indirectly through Tregs. A second possibility is that CTLA-4 may function to skew the T cell repertoire. However, based on previous studies by Allison and colleagues, it would be expected that, if anything, the repertoire would be broadened to include lower affinity clones that would escape CTLA-4 inhibition due to decreased action of the inhibitory receptor (36). A third possibility is that, over time, CTLA-4–resistant T cell clones emerge by overcoming the threshold of CTLA-4–mediated inhibition and become less dependent on costimulation. Autoimmune diabetes is a chronic progressive disease with autoreactive T cells accumulating within the target organ months prior to clinical manifestations (35). Given the disease kinetics, it is possible that autoreactive cells may develop mechanisms to overcome or bypass CTLA-4–mediated suppression. This idea is supported by the work of You et al., where equal frequencies of diabetogenic T cells were detected in 6-week-old prediabetic and overtly diabetic NOD mice (37). They proposed that the progression to overt disease correlates with a qualitative change in the pathogenic T cells that allows them to escape from regulation over time (37). Thus, in a chronic progressive autoimmune disease, such as type 1 diabetes, evolution of pathogenic T cells is likely to post significant challenge in designing novel immunosuppressive therapies.

Overall, we have demonstrated anti–CTLA-4 Abs anchored on the surface of APCs to be an effective means to suppress autoimmune responses. There are a number of ways that can be envisioned to adapt such an approach in clinical settings to prevent graft rejection and treat autoimmunity. The first is a gene therapy approach targeting APCs, including macrophages, DCs, and B cells, to express this construct with the intent that antigen-specific responses would be selectively engaged together with CTLa-4 inhibition. A second approach shown to be effective for targeting autoimmunity was the use of a bispecific Ab to simultaneously engage antigen and CTLA-4 (38). This approach was effective in a mouse model of thyroiditis using bispecific Abs against thyroid-stimulating hormone receptor and CTLA-4 (39). The major limitation for this strategy is the requirement of identifying and generating tissue-specific Abs against immunodominant antigens for each disease. The last approach to selectively target autoreactive and
'allogeneic' T cells is to modify the membrane of APCs or grafted tissue with inhibitory molecules. Recent reports have demonstrated effective use of cell membrane-anchored FasL to prevent islet and cardiac graft rejection (40). This approach utilizes a protocol to biotinylate cells and "decorate" the membrane with a chimeric protein comprised of streptavidin and FasL (40). This is an attractive approach for the clinical setting for treatment of autoimmune disease and prevention of graft rejection. Patient cells, including B cells, macrophages, DCs and even islet grafts, could be biotinylated and modified with the addition of streptavidin scFvCTLA-4. All of these therapeutic approaches are aimed toward developing a system in which antigen-specific inhibition using APCs is combined with CTLA-4-mediated inhibition.

Our results demonstrate profound inhibition of antigen-specific T and B cell responses by scFvCTLA-4 Tg B cells both in vitro and in vivo. Moreover, we show that selective engagement of CTLA-4 conferred significant protection against an exacerbated form of autoimmune diabetes. These results suggest that simultaneous engagement of CTLA-4 during antigen presentation may be an effective regimen for treating autoimmune diseases and organ graft rejections. To this end, we have focused on the generation of a construct that results in deliberate ligation of CTLA-4 in vivo and represents what we believe to be a novel immunotherapeutic approach. This type of antigen-specific approach may be useful for treating autoimmunity and inducing peripheral tolerance and warrants further development and consideration.

Methods

Mice. Female FVB, C57BL/6, and C3H mice were purchased from Jackson Laboratory. Female NOD mice were purchased from Taconic. NOD.BDC2.5 TCR Tg mice (27) were generously provided by C. Benoist and D. Mathis (Harvard Medical School, Boston, Massachusetts, USA), and C57BL/6.CTLA-4 KO mice were generously provided by A. Sharpe (8) (Harvard Medical School, Boston, Massachusetts, USA). IFN-γ reporter mice were generated on the C57BL/6 background and backcrossed 12 generations to NOD and subsequently bred to NOD.B7 DKO for 10 generations. Subsequently, the NOD.scFvCTLA-4 Tg mice were crossbred to NOD.B7 DKO mice.

Antigens. DNP-OVA and DNP–keyhole limpet hemocyanin (DNP-KLH) were purchased from Genemed Synthesis Inc. The amino acid composition was verified by mass spectrometry, and purity (>98%) was assessed by HPLC.

Cell sorting. scFvCTLA-4 Tg B cells were stained with the fusion protein mCTLA-4 Ig (Genetics Institute Inc.) and anti-CD2-M1 (Genetix) and anti-CD4-FITC (Pharmingen) and with anti-B220, in the presence of anti–B7-1 and anti–B7-2 to block endogenous B7a. B220+ CTLA-4+ IgG1+ cells were sorted using a MoFlo cytometer high speed cell sorter (Dako). NOD.BDC2.5 Thy1.1 cells were stained with anti-CD4 FITC, anti-CD25 PE, and anti-CD62L APC, and the CD4+CD62L−CD25− T cells were sorted. All sorted populations had greater than or equal to 98% cell purity.

Activation of donor lymphocytes, cell culture, and transfer. MoFlo-sorted WT FVB and scFvCTLA-4 Tg B cells were activated by LPS (1 μg/ml, Sigma–Aldrich) in vitro for 72 hours, loaded with DNP-OVA and incubated for 4 hours at 37°C, and transferred to naive FVB recipients. Seven days following transfer of cells, recipient animals were euthanized subcutaneously in the flank with DNP-OVA (100 μg/mouse) in CFA (Sigma–Aldrich) containing 4 mg/ml Mycobacterium tuberculosis (BD Diagnostics).

Murine cytomegalovirus infection. NOD, NOD.scFvCTLA-4 Tg, and NOD RAG KO mice were infected with 5 × 10⁶ PFU CMV Smith strain. Fourteen days after infection, liver and spleen tissue was harvested, weighed, and homogenized, and viral plaque assays were performed using NIH 3T3 mouse embryonic fibroblast cells using serial dilutions.

Assessment of insulin auto-Ab. Serum samples were collected from 5-, 10-, 20-, and 30-week-old NOD.scFvCTLA-4 Tg and NOD control mice (n = 7–10/time point/group), and insulin auto-Ab were determined as previously described (41).

Assessment of diabetes and insulin. Blood glucose levels were measured from female NOD, NOD.scFvCTLA-4 Tg, NOD.B7 DKO, and NOD.B7 DKO. scFvCTLA-4 Tg mice weekly with an ACCU-CHEK Active glucose meter (Roche Diagnostics). Mice were considered diabetic with measurements over 250 mg/dl. For histological analysis, pancreas was formalin fixed in 10% buffered formalin. Multiple 5-μm sections were stained with H&E and scored blindly for severity of insulinitis as previously described (26).

CFSE adoptive transfer. To measure in vivo proliferation, LN cells from NOD.B7 Thy1.1 mice were depleted of CD25+ cells using anti-CD25 (clone 7D4) and complement, followed by Ficoll density gradient centrifugation. Cells were labeled with 2.5 μM CFSE (Invitrogen), and 1 × 10⁵ cells were intravenously transferred to recipient mice. Recipients were sacrificed 5 days later, and CFSE dilution of pancreatic and inguinal LN cells was measured by flow cytometry using a BD LSRII cytometer (BD Biosciences — Pharmingen).

ELISA. Assessment of cytokine production was tested for IL-2, IL-4, IL-10, and IFN-γ by commercial ELISA kits according to manufacturer-recommended protocol (Endogen, Pierce Biotechnology). Total Ig, IgG2a, IgG1, and IgM anti-DNP Ab production was measured at days 0, 7, and 14 following transfer of antigen-loaded WT and scFvCTLA-4 Tg B cells. In brief, flat-bottom microtiter plates (Nunc) were coated with DNP-KLH (50 μg/ml) in PBS coating buffer and blocked with 2% BSA (Sigma–Aldrich) in PBS for 1 hour at room temperature; serum samples were subsequently added in triplicate. Biotinylated anti-Ig, anti-IgG2a, anti-IgG1, and anti-IgM (BD Biosciences — Pharmingen) were added and incubated for 2 hours. Plates were developed using streptavidin-peroxidase (Zymed) and OPD substrate (Sigma–Aldrich), and absorbance was read at 405 nm using a Vmax kinetic microplate reader ( Molecular Devices).

In vivo T cell proliferation assays. Cells were cultured in 96-well microtiter plates (Corning Inc.) at 5 × 10⁴ viable cells/well in complete DMEM. Cells
were pulsed with 1 μCi of 3H-TdR (ICN Radiochemicals) during the last 8 hours of a 96-hour culture, and 3H-TdR uptake was detected using a Packard TopCount Microplate Scintillation Counter (Packard Instruments Co.).

Statistics. Statistical significance of cytokine levels, thymidine incorporation, and mean time to 25% disease incidence was analyzed using 2-tailed Student’s t test for comparisons of 2 means. Diabetes incidence statistical significance was analyzed using a nonparametric Wilcoxon signed-rank test. Values of \( P \leq 0.05 \) were considered significant.

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