Opposing Effects of CTLA4 Insufficiency on Regulatory versus Conventional T Cells in Autoimmunity Converge on Effector Memory in Target Tissue

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Quantitative variations in CTLA4 expression, because of genetic polymorphisms, are associated with various human autoimmune conditions, including type 1 diabetes (T1D). Extensive studies have demonstrated that CTLA4 is not only essential for the suppressive role of regulatory T cells (Treg) but also required for intrinsic control of conventional T (Tconv) cells. We report that a modest insufficiency of CTLA4 in mice, which mimics the effect of some human CTLA4 genetic polymorphisms, accompanied by a T1D-permissive MHC locus, was sufficient to induce juvenile-onset diabetes on an otherwise T1D-resistant genetic background. Reduction in CTLA4 levels had an unanticipated effect in promoting Treg function both in vivo and in vitro. It led to an increase in Treg memory in both lymphoid and nonlymphoid target tissue. Conversely, modulating CTLA4 by either RNA interference or Ab blockade promoted conventional effector memory T cell formation in the Tconv compartment. The CD4+ conventional effector memory T cells, including those within target tissue, produced IL-17 or IFN-γ. Blocking IL-7 signaling reduced the Th17 autoimmune compartment but did not suppress the T1D induced by CTLA4 insufficiency. Enhanced effector memory formation in both Tconv and Treg lineages may underpin the apparently dichotomized impact of CTLA4 insufficiency on autoimmune compartment but did not suppress the T1D induced by CTLA4 insufficiency. Enhanced effector memory formation in both Tconv and Treg lineages may underpin the apparently dichotomized impact of CTLA4 insufficiency on autoimmune pathogenesis. Therefore, although the presence of CTLA4 plays a critical role in controlling homeostasis of T cells, its quantitative variation may impose diverse or even opposing effects on distinct lineages of T cells, an optimal sum of which is necessary for preservation of T cell immunity while suppressing tissue damage. The Journal of Immunology, 2014, 193: 4368–4380.

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ne of the hallmarks of the adaptive immune response is its ability to generate immunological memory against foreign Ags so as to generate a stronger immune response on secondary encounter. However, when an immune response is generated against self-Ags, immunological memory is often thought to contribute to the persistence of the autoimmune response in autoimmune diseases (1). It remains unclear how the different memory T cell subsets contribute to autoimmune damage in the target tissues.

Recent studies have shown that the basic tenets of immunological memory apply to the regulatory T cell (Treg) compartment as well. During acute viral infections, it has been shown that Tregs go through the classical Ag-specific expansion, contraction and memory maintenance phases (2, 3). On secondary rechallenge, these memory Tregs are able to suppress conventional T cell (Tconv) responses better than naive Tregs, thus helping to prevent excess immunopathology during a recall response. In a model of skin autoimmunity, it has been shown that memory Tregs in the tissue confer superior protection against autoimmune attack (4). Thus, Treg memory in the tissue is emerging as one of the main players in regulation of autoimmune responses where persistent self-Ag expression helps in its maintenance (5).

A complex interplay of genetic factors often influences the onset of autoimmune diseases. In humans, one of the most important genetic contributors to type 1 diabetes (T1D) identified so far has been the HLA haplotype of the individual (6). Most often this genetic risk conferred by the HLA haplotype is influenced by non-HLA genetic factors such as CTLA4 genetic polymorphisms. CTLA4 is a negative regulator of the immune system (7). CTLA4 gene polymorphisms have also been implicated in a number of autoimmune disorders (8). Most disease-associated single nucleotide polymorphisms (SNPs) of CTLA4 have been mapped to the noncoding regions, such as promoter and 3′-untranslated region polymorphisms (9–12). These do not result in an ablation of CTLA4 production but rather result in a modest reduction in levels of functional CTLA4 protein (9–12) or alter the ratios of the various CTLA4 splice variants (13).

CTLA4 is expressed as multiple splice variants (7). Studies by several groups have established the function of each splice variant in various autoimmune settings (13–17). Nevertheless, the exact impact of each CTLA4 polymorphism on T1D remains a debate. For example, one study showed that unstimulated CD4 T cells from 14 healthy subjects had ~2- to 3-fold lower levels of soluble CTLA4, an effect associated with the T1D-risk +6230G alleles (13). However, a later study with 11 nondiabetic subjects including parents of T1D children did not find the linkage of +6230G > A SNP to either soluble CTLA4 or full-length CTLA4.
levels if the subjects had the same −318C SNP in the promoter region of the CTLA4 gene, but the −318C T1D-risk allele was associated with lower levels of both full-length CTLA4 and soluble CTLA4 expression (18). The discrepancy could be because of diverse ethnicity, environmental, or other factors. In contrast, the many studies associating the CTLA4 locus with T1D have suggested a consensus theme: there is no qualitative change of mature CTLA4 protein; instead, it is the modest quantitative reduction of CTLA4 that may pose a genetic risk for T1D. However, the exact impact of such quantitative changes on immune cells during T1D development remains to be characterized, especially in a disease model that reflects the human T1D onset at a juvenile age with a natural immune cell repertoire, besides the standard NOD model that has adulthood-onset diabetes with gender bias.

To model the effect of such a modest reduction in CTLA4 expression on T1D pathogenesis, we used a CTLA4RNAi mouse model (19–21). This model enabled us to study the specific influence of a modest reduction in CTLA4 coupled to a disease-susceptible MHC on spontaneous development of T1D by crossing the CTLA4RNAi transgene onto the B6.H2<sup>β<sub>2</sub></sup> background. B6.H2<sup>β<sub>2</sub></sup> mice harbor the T1D-susceptible MHC loci from the NOD strain but with a genetic background of wild-type C57BL6 mice (22). This new model, with diabetes penetrance at juvenile age, allowed us to examine autoimmune memory T cells in target tissue during onset of T1D at young age in the animal.

In acute infectious disease settings, the CD62L<sup>lo</sup>CD44<sup>hi</sup> population is presumed to represent the effector memory T cell population long after Ag clearance because effector T cells are activated or systemic inflammation responses (19). We did not find significant differences between the PL4 transgenic line on the B6.H2<sup>β<sub>2</sub></sup> genetic background as additional controls, we did not generate a new line of PL4.B6.H2<sup>β<sub>2</sub></sup> mice because of the costs involved. The transgene-negative littermate controls, as discussed previously, were deemed adequate because the PL4 transgenic line did not promote autoimmune damage to the pancreas in the NOD model (19), in the BDC2.5/NOD model or on the B6 genetic background (see Results below).

BDC2.5/NOD.Foxp3<sup>GFP</sup> mice were generated by crossing BDC2.5/NOD with NOD.Foxp3<sup>GFP</sup> mice (20, 21). The CTLA4RNAi transgenic line carries a lentiviral short hairpin RNA transgene targeting a 3′-untranslated region shared by all splice variants of CTLA4. It reduced CTLA4 expression 2- to 3-fold in both the T<sub>reg</sub> and T<sub>conv</sub> compartments (19). IL-17A knockout B6.IL17<sup>−/−</sup> mice (29) (The Jackson laboratory) were crossed with CTLA4RNAi/B6 mice to generate CTLA4RNAi/B6.IL17<sup>−/−</sup> mice. The studies were approved by the Institutional Animal Care and Use Committee at the University of Miami. All animals were maintained in a specific pathogen-free barrier facility.

Diabetes monitoring and insulin scoring

Monitoring of diabetes incidence by urine and blood glucose measurement and assessment of immune damage in the pancreatic islet by histopathology examination were conducted according to standard procedures (21, 28).

Liver histology scoring

Inflammatory infiltration was scored based on the extent of infiltration around blood vessels (including the central vein, portal vein, and the hepatic artery) and sinusoids. Each blood vessel/sinusoid was scored for inflammatory infiltration into it, and the total score was averaged to assign a score to the liver (0, no lymphocytic infiltration; 2, light infiltration; 4, medium infiltration; and 6, heavy infiltration).

Isolation of cells from the pancreas

Mouse pancreata were cut into small pieces (<2 mm) and incubated in RPMI 1640 medium containing 55% FBS in a petri dish at 37°C with 5% CO<sub>2</sub> for 3–4 h immediately after dissection from the mouse. The mouse was either perfused with PBS, or the pancreatic tissue was blotted and washed with PBS to remove lymphocytes from the blood circulation. After 3–4 h, the pancreatic debris was removed, and the cells in the medium were collected from the petri dish, filtered with a 70-μm cell strainer, and analyzed.

Flow cytometry

Single-cell suspensions of spleens and lymph nodes were prepared for staining. The cells were then blocked using anti-CD16/32 (2.4G2) and normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were then stained for surface markers with Ab conjugates. The following fluorescently labeled mAbs were used: CD4-PECy7, CD8-Brilliant Violet 605, CD44-Brilliant Violet 785, CD127-biotin, CD45-biotin (BioLegend), IL-2-mCherry (BioLegend), and IL-7-mCherry (BioLegend). Staining for intracellular Foxp3+ staining, cells were fixed and permeabilized with reagents in the Foxp3 staining kit (eBioscience) and then stained intracellularly with Foxp3-eFluor560 (eBioscience). Cells were then analyzed using a LSR II flow cytometer with FACS Diva software (BD Biosciences).

For intracellular cytokine staining, single-cell suspensions of cells were prepared in complete RPMI 1640 medium. Four million cells per milliliter were resuspended in stimulation medium (complete RPMI 1640 medium + 40 nM PDBu + 2 μM ionomycin) and incubated at 37°C for 6 h. Brefeldin A (eBioscience) was added 30–60 min after beginning of incubation. The cells were then collected and stained for surface markers. The cells were then fixed with 2% PFA for 30 min. This was followed by permeabilization using the perm/wash buffer (BD Biosciences). For intracellular cytokine staining, the following Abs were used: IFN-γ-PECy7 (BioLegend), IL-
CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec, San Diego, CA) was used to isolate CD4+ Treg and Tconv. 100,000 Tconv were stimulated with 100,000 irradiated APCs and 0.5 μg/ml anti-CD3 (eBioscience) for 72 h in a 96-well round-bottom tissue culture plate. The culture was pulsed with [3H]thymidine for the last 16 h. The cellular cytokine profiles, cell populations were first gated on CD3+CD8+ (for CD8+ T cells) and then analyzed for TEMs (CD44hiCD127+) and TEFFs (CD44hiCD127loCD69+). Treg compartment was analyzed for Tconv proliferations using the red fluorescent protein marker. Naive Treg (CD4+CD25−CD69−Foxp3−) were sorted by FACS on the B6.129S1-Cd4tg1 Tcrd Tcrd mice background. Early naive Treg were transferred into NOD.SJL-Ptprc ZM1/Bom congenic mice. For adoptive transfer studies of TEMs and TEFFs of the Tconv compartment, donor spleen cells from BDC2.5/NOD/Foxp3−/− mice were processed using the method described above. CD4+ TEMA, or CD4+ TEMB, were sorted using a FACS Aria II flow cytometer (BD Biosciences). Fifty thousand sorted CD4+ TEMA, or CD4+ TEMB, from donor mice were injected i.v. administered with anti–IFN-γ (clone XMG1.2) Ab or rat IgG isotype control Ab (BioXCell, West Lebanon, NH) at a dose of 25 μg/kg. These mice were then monitored for diabetes and at the end point were analyzed by flow cytometry. For lymphoproliferate transfer experiments, 200,000 CD4+ TEMA, or CD4+ TEMB, sorted from BDC2.5/NOD/Foxp3−/− mice were injected i.v. into adult CD90.1 congenic NOD recipients. These mice were sacrificed 5–6 d after transfer and were analyzed by flow cytometry.

T reg in vitro suppression assay

CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec, San Diego, CA) was used to isolate CD4+ Treg and Tconv. 100,000 Tconv were stimulated with 100,000 irradiated APCs and 0.5 μg/ml anti-CD3 (eBioscience) for 72 h in a 96-well round-bottom tissue culture plate. Isolated Treg cells were titrated at Treg/Tconv ratios of 1:1, 1:2, 1:4, 1:8 and 1:16. The culture was pulsed with [3H]thymidine for the last 16 h. The amounts of incorporated 3H were measured by a beta counter (1450 LSC and Luminescence counter; PerkinElmer, Boston, MA). Suppression was calculated as the % reduction in CPM compared with the Tconv proliferation on stimulation with no Treg cells added.

Ab treatment

Anti-CTLA4 (UC-10) and anti-IL-7R α (IL-7Rα, CD127) (clone A7R34) (23) Abs for in vivo blocking experiments were purified from hybridoma cell culture. BDC2.5/NOD/Foxp3−/− mice were i.p. administered with anti-CTLA4 (UC-10) Ab or hamster IgG control Ab (Rockland Immunochemicals, Gilbertsville, PA) at a dose of 30 μg/kg body weight on days 8 and 11 after birth. The flow cytometry analyses were done at 3 wk of age. For in vivo studies, B6.129S1-Cd4tg1 Tcrd Tcrd mice with B6.H2−/− mice were i.p. administered with anti–IL-7R (A7R34) Ab or rat IgG isotype control Ab (BioXCell, West Lebanon, NH) at a dose of 25 μg/kg body weight beginning at 3–4 d of age, twice a week for 3 wk. These mice were then monitored for diabetes occurrence every 2–3 d until diabetes onset or for 6 wk.

Statistics

Log-rank (Mantel–Cox) test was used for cumulative diabetes incidence. Student t tests were used for single comparisons (mean ± SEM). For the cells numbers in the pancreas of anti–CTLA4-treated BDC2.5/NOD mice, Mann-Whitney U test was used to test for significance because the variability of cell numbers in the tissue of young mice. A P value < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01, and ***P < 0.005.
A modest insufficiency of CTLA4 increased the suppressive ability of Tregs in vitro, with CTLA4RNAi Tregs exhibiting a significant increase in suppressive ability (Fig. 2E). Taken together, these experiments suggest that a reduction in CTLA4 levels may enable Tregs to become better suppressors in vivo and in vitro. Enhanced suppressive ability of Tregs was accompanied by an increased regulatory effector memory phenotype. Superior suppression mediated by Tregs with a reduction in CTLA4 levels could be attributed to the impact of CTLA4 reduction on their differentiation or activation. To explore this possibility, we analyzed the phenotype of the Treg populations affected by CTLA4RNAi in mice on the T1D-susceptible (B6.H2g7) or -resistant (B6) backgrounds in the pancreas and the lymph nodes (Fig. 3A–C). Flow cytometry analyses showed that there was an increase in the total Treg population in CTLA4RNAi mice on either the B6.H2g7 or the B6 background (data not shown). These Tregs were also phenotypically more memory-like, with a substantial increase in the total number of Treg-EMs in the target tissue (Fig. 3A–C). It has been recently shown that Treg-EMs are able to confer superior protection in the tissue by being able to respond to autoimmune attack faster than Treg-EFFs (4). Thus, the increased number of Treg-EMs in the pancreas of the CTLA4RNAi model would imply superior regulation by the Tregs at the site of ongoing autoimmunity.

A polyclonal T cell repertoire system such as the CTLA4RNAi/B6.H2g7 and CTLA4RNAi/B6 models represent the natural T cell repertoire in humans; however, it is often difficult to pinpoint the response of Ag-specific cells. To study the effect of CTLA4RNAi on Ag-specific Tregs in autoimmune damage, we used the BDC2.5/NOD model, a well-characterized MHC class II–restricted TCR transgenic mouse model of T1D where the CD4+ T cells specifically recognize a natural β-cell autoantigen (34). Flow cytometry analyses of the pancreas of these mice revealed that a majority of the activated autoantigen-specific Treg population was phenotypically effector memory like and the proportion of the Treg-EM population in the pancreas was 3-fold higher when compared with the draining lymph nodes (PLN) (Fig. 3D, 3E). In contrast, the activated Tregs in the PLN primarily were made up of Treg-EFFs (Fig. 3D, 3E). Tregs in the pancreatic infiltrate of BDC2.5/NOD mice have been shown to be primarily responsible for suppressing tissue damage by the infiltrating autoimmune Tconvs (28). Therefore, the increased proportion of Ag-specific Treg-EMs in the pancreas of BDC2.5/NOD mice emphasizes the importance of this subset in conferring superior protection at the forefront of autoimmune damage in the tissue. CTLA4 reduction further increased the Ag-specific Treg-EM population in the pancreas and PLNs (Fig. 3F), suggesting enhanced Treg regulation at the site of ongoing autoimmunity in a setting of reduced CTLA4 expression. Thus, a modest reduction in CTLA4 levels may lead to decreased intrinsic control of Tregs, resulting in their increased effector memory-like characteristics that potentiate their increased suppressive ability. This outcome is opposite to what was expected based on the effect of a complete ablation of CTLA4 in Tregs (31),...
suggesting that a modest reduction in CTLA4 levels, as detected in some human patients with an increased susceptibility to T1D, may lead to an effect on Treg function opposite to that of complete blockade of CTLA4.

**Predominance of CD4+ effector memory cells in the Tconv compartment in the pancreas was associated with autoimmune diabetes**

Previous studies have shown a complexity of CTLA4 function in regulating the Tconv compartment by both cell-extrinsic and cell-intrinsic mechanisms (35–37). Because the increased susceptibility to T1D by CTLA4RNAi in the B6.H2g7 background was not due to a defective Treg compartment, we investigated the effects of CTLA4RNAi on the Tconv compartment.

To study the Tconv compartments including TCMs, TEMs, and TEEFs, surface markers CD44, CD62L, CD69, and CD127 were used to analyze these subsets in the PLN and pancreas (Fig. 4A). Within the CD8+ T cell compartment, there was little activation observed in the PLNs (Fig. 4A). The percentage of CD8+ TEM in the pancreas was ~10% of the total CD8+ population (Fig. 4A, 4B). However, there was no significant difference between CTLA4RNAi and control mice in this population. These two groups had no difference in the percentage of CD8+ TEEFs (Fig. 4C) or CD8+ TCMs (Fig. 4D) either.

Unlike the CD8 compartment, analyses of the CD4 compartment revealed a substantial increase in the CD4+ TEM in the pancreas of CTLA4RNAi/B6.H2g7 mice compared with controls (Fig. 5A–E). The highest proportion of CD4+ TEM was found in the pancreas (Fig. 5B, 5E), with an additional 2-fold increase in their proportion in the pancreas of CTLA4RNAi/B6.H2g7 mice compared with controls (Fig. 5B). This increase in proportion was also reflected as an increase in the total number of CD4+ TEMs in the pancreas of CTLA4RNAi/B6.H2g7 mice (Fig. 5B). The increase in the autoimmune CD4+ TEM population in the pancreas of CTLA4RNAi/B6.H2g7 mice was also accompanied by an increase in the total numbers of CD4+ TEEFs in the target tissue (Fig. 5C). However, compared with the high percentage of the CD4+ TEM population (average 30%) in the CD4+ Tconv compartment in the pancreas, the CD4+ TEEF population is only a minor subset (average 2%) in the tissue of CTLA4RNAi/B6.H2g7 mice. There was no difference observed in the CD4+ TCM compartment in the tissue of CTLA4RNAi/B6.H2g7 mice compared with the controls (Fig. 5D). Similar increases in the CD4+ TEM subset were detected in the pancreas of CTLA4RNAi/B6 mice (Fig. 5E).

TCMs are thought to reside in lymph nodes. We analyzed the TCM compartment in the PLN and found that there was an increase in the proportion of CD4+ TCM in the PLN of the CTLA4RNAi/B6.H2g7 mice over controls (Fig. 5D). However, constant persistence of self-Ags may be untoward to the development of TCMs, akin to chronic infection settings (38). Thus, it remains unknown whether this population of T cells with the central memory phenotype in the lymph nodes of CTLA4RNAi/B6 mice has a pathogenic potential.

Akin to CTLA4, PD-1 is another member of the CD28 family of receptors that has been shown to play an important role in T cell regulation and the dysregulation of which has been implicated in autoimmune diabetes (39–41). We analyzed PD-1 expression on the CD4+ TEM and CD4+ TEEF compartments in B6.H2g7 mice (Fig. 5F, 5G). A substantially greater proportion of CD4+ TEEFs expressed PD-1 when compared with the CD4+ TEMs (Fig. 5G). This implied that the autoimmune CD4+ TEM compartment could be more resistant than the CD4+ TEEF compartment to exhaustion mediated by the PD-1 pathway. A reduction in CTLA4 levels in B6.H2g7 mice, however, had no significant impact on PD-1 expression in the CD4+ TEM and CD4+ TEEF subsets (Fig. 5H).
CTLA4 reduction promoted autoantigen-specific CD4+ TEM in the Tconv compartment in target tissue

To characterize the memory phenotype of autoantigen-specific cells in the Tconv compartment, we used the BDC2.5/NOD model (34). The known Ag specificity of the T cells against pancreatic β cells allows us to test the diabetogenic potential of the autoimmune CD4+ TEM and TEFF cells. We purified the two subsets from BDC2.5/NOD.Foxp3FIR spleens and adoptively transferred them into NOD.SCID mice. Indeed, the TEMs but not the TEFFs, caused diabetes efficiently (Fig. 6A), demonstrating that the CD4+ TEM compartment has a much higher pathogenic potential than the CD4+ TEFF compartment. Flow cytometry analyses revealed a comparable proportion of CD4+ T cells in the NOD.SCID mice transferred with the CD4+ TEMs or CD4+ TEFFs, in the PLN (Fig. 6B), and in other lymphoid organs (data not shown), implying that the heightened autoimmune pathogenicity of CD4+ TEMs over the CD4+ TEFF subset was not due to an advantage of expansion and/or survival of the CD4+ TEM subset over the CD4+ TEFF subset in the lymphopenic environment in NOD.SCID mice. To compare the autoantigen-specific responses of these subsets in a lymphoreplete system, the CD4+ TEM or the CD4+ TEFF subsets were purified from BDC2.5/NOD.Foxp3FIR mice and were adoptively transferred into CD90.1 congenic NOD mice. Flow cytometry analyses revealed that more of the transferred CD4+ TEMs

FIGURE 3. CTLA4 reduction increased effector memory formation of Treg in the target tissue of mice on diverse genetic backgrounds. (A) Flow cytometry analyses of the Treg-EFF and Treg-EM subsets in the pancreas (numbers represent percentages of gated Treg populations). (B) Frequencies and total cell numbers of the CD4+ Treg-EM subset with CTLA4RNAi or transgene-negative littermate controls on the B6.H2d7 background (n = 5–6/group, 4–12 wk old; mean ± SEM). (C) Analyses of mice on the B6 background. Control data represent a pool of transgene-negative littermates or age- and sex-matched PL4 vector transgenic mice (n = 4/group, 9–16 wk old; mean ± SEM). (D) Flow cytometry plots of the PLN and pancreas in BDC2.5/NOD mice showing autoantigen-specific Treg-EFF and Treg-EM subsets (numbers represent percentages of gated Treg populations). (E) Comparison of the frequencies of Ag-specific CD4+ Treg-EM and CD4+ Treg-EFF subsets in the tissue and the draining lymph nodes of BDC2.5/NOD mice (n = 7/group; line indicating average of the group). (F) Frequencies and total cell numbers of the autoantigen-specific Treg-EM subset affected by CTLA4 modulation. Control data represent a pool of CTLA4RNAi-transgene–negative littermate BDC2.5 mice or age- and sex-matched PL4/BDC2.5 mice (n = 6–7/group, 7–12 wk old; mean ± SEM). Each data point represents one animal. *p < 0.05, **p < 0.01, ***p < 0.005.
TCM, T Eff, and TEM subsets of the conventional CD8+ T cell compartment play a key role in T1D pathogenesis. Furthermore, the effect of CTLA4 modulation on the TEM compartment was also evident with anti-CTLA4 Ab blockade (Supplemental Fig. 2).

Increased production of IL-17 and IFN-γ by the pathogenic autoimmune CD4+ TEM compartment because of a reduction in CTLA4 levels

Persistence of Ags in chronic infectious diseases has been shown to result in exhausted T cells that produced reduced levels of cytokines. However, despite persistence of self-Ags in the context of autoimmune diseases, the production of IL-17 and IFN-γ by T cells has been implicated in the pathogenesis of various autoimmune diseases including T1D (42).

To characterize the production of pathogenic cytokines by the autoimmune T cell compartment, we generated CTLA4RNAi transgenic mice on the B6.H2g7 background. Control mice were transgene-negative littermates. (Fig. 6A). Frequencies and total cell numbers of CD8+ TEM and T Eff subsets impacted by CTLA4RNAi on the B6.H2g7 background. Control mice were transgene-negative littermates (n = 6/group, 4–12 wk old). Each data point represents one animal (mean ± SEM).

could be detected in the draining lymph nodes of the recipient mice when compared with that in the animals transferred with CD4+ T Effs (Supplemental Fig. 1).

To study the effect of reduced levels of CTLA4 on this pathogenic CD4+ TEM compartment, we generated CTLA4RNAi/BDC2.5 mice or PL4/BDC2.5 controls by crossing the CTLA4RNAi/NOG model with the BDC2.5/NOG line. The 60% reduction in CTLA4 levels resulted in 100% of the mice becoming diabetic by 12–16 wk of age, whereas no diabetes was detected up to this age in the untreated CTLA4RNAi/B6.H2g7 mice as shown in Fig. 1A. The treatment efficiently blocked IL-7Rα treatment when compared with the isotype control treatment (Fig. 8D). In the anti–IL-7Rα–treated group, 37% of CTLA4RNAi/B6.H2g7 mice became diabetic at 21–26 d of age, which is similar to the 30% diabetes incidence in untreated CTLA4RNAi/B6.H2g7 mice as shown in Fig. 1A. However, there was a 3-fold decrease in the proportion of IL-17–producing CD4+ T cells (Fig. 8C) in the anti–IL-7Rα–treated group compared with PL4/BDC2.5 controls (Fig. 8C) in the draining lymph nodes (PLN). This amounted to a 7-fold increase in the total numbers of Th17 TEM subset producing IFN-γ, with a greater proportion of the PLN TEM subset producing IFN-γ when compared with the PLN T Eff subset (Fig. 7B). Clearly, compared with the IFN-γ and IL-17 production in the PLN, the production of these cytokines by the autoantigen-specific CD4+ TEM was not impeded in the pancreas despite persistence of self-Ags in the target tissue (Fig. 7A, 7B).

We further analyzed the impact of CTLA4 modulation on cytokine production by the autoimmune CD4+ TEMs and CD4+ T Effs in the PLN and pancreas (Fig. 7C–H). Intracellular cytokine staining revealed a 2-fold increase in the total number of IL-17–producing CD4+ TEMs in the pancreas of CTLA4RNAi mice compared with controls (Fig. 7C). CTLA4 reduction also led to a 3-fold increase in the total number of IFN-γ–producing autoimmune CD4+ TEMs (Fig. 7E) and a 2-fold increase in the IFN-γ–producing autoimmune CD4+ T Effs in the pancreas (Fig. 7G). The impact of CTLA4RNAi on Th1 and Th17 memory was similar to that of CTLA4 blockade by mAb treatments. Anti-CTLA4 treatment in young BDC2.5/NOG mice resulted in an increase in the total numbers of Th17 TEMs (Fig. 7D) and Th1 TEMs (Fig. 7F) in the PLN and pancreas.

Anti–IL-7Rα treatment reduced Th17 memory in the tissue but without substantial effect on T1D development

On the B6.H2g7 background, a reduction in CTLA4 levels led to a 3-fold increase in the percentage of IL-17–producing CD4+ T cells (Fig. 8A, 8B) and a 2-fold increase in the percentage of IFN-γ–producing CD4+ T cells (Fig. 8C) in the draining lymph nodes (PLN). This amounted to a 7-fold increase in the total numbers of IL-17–producing CD4+ T cells (Fig. 8B) and a 4-fold increase in the total numbers of IFN-γ–producing CD4+ T cells (Fig. 8C) in CTLA4RNAi/B6.H2g7 mice compared with the controls.

Given the prominent role of IL-7 in T cell memory development (43, 44), we used mAbs against IL-7Rα (CD127) to inhibit diabetes development in the juvenile-onset T1D model. We treated CTLA4RNAi/B6.H2g7 mice with anti–IL-7Rα Ab for 3 wk after birth. The treatment efficiently blocked IL-7Rα (Supplemental Fig. 3). However, there was no significant decrease in diabetes incidence on anti–IL-7Rα treatment when compared with the isotype control treatment (Fig. 8D). In the anti–IL-7Rα–treated group, 37% of CTLA4RNAi/B6.H2g7 mice became diabetic at 21–26 d of age, which is similar to the 30% diabetes incidence in untreated CTLA4RNAi/B6.H2g7 mice as shown in Fig. 1A. However, there was a 3-fold decrease in the proportion of IL-17–producing CD4+ T cells in the pancreas and draining lymph nodes.
FIGURE 5. CTLA4 reduction increased CD4+ effector memory formation in the Tconv compartment. (A) Flow cytometry analyses of the naive, Tconv, Tconv, and Tconv subsets of the conventional CD4+ T cell compartment in the PLN and pancreas (numbers represent percentages of gated CD4+ Tconv populations). (B–D) Frequencies and total cell numbers of CD4+ Tconv, CD4+ Tconv, and CD4+ Tconv subsets affected by CTLA4RNAi on the B6.H2b7 background. Control mice were transgene-negative littermates (n = 5–7/group, 4–12 wk old). (E) Frequencies and total cell numbers of CD4+ Tconv, affected by CTLA4RNAi on the B6 background. Control data represent a pool of transgene-negative littermates or age- and sex-matched PL4 vector transgenic mice (n = 4/group, 9–16 wk old). (F) Flow cytometry analyses of the PD-1 expression by CD4+ Tconv and CD4+ Tconv subsets (numbers represent percentages of gated CD4+ Tconv populations). The first two plots show staining with an isotype control Ab of the anti–PD-1 Ab. (G) The frequencies of PD1+ cells in the CD4+ Tconv and CD4+ Tconv subsets in B6.H2b7 mice. (H) Effects of CTLA4 reduction on the frequencies of PD1+CD4+Tconv and PD1+CD4+Tconv subsets on the B6.H2b7 background. Control mice were transgene-negative littermates (n = 4/group, 12–17 wk old). Each data point represents one animal (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.005.
This effect was sustained for 5–6 wk after the last treatment. In contrast, there was no significant reduction in the proportion of IFN-γ–producing CD4+ T cells in the pancreas of anti–IL-7Rα Ab–treated mice (Fig. 8E–G). The reduction in total numbers of IFN-γ–producing cells in the lymph nodes (Fig. 8G) could be attributed to the overall reduction in total T cell numbers caused by the anti–IL-7Rα treatment, as has been shown in other studies as well (23, 24). The reduction in the percentage of the Th17 subset but not that of the Th1 subset suggests a different mechanism of action of anti–IL-7Rα Ab treatment in the juvenile-onset T1D model when compared with adulthood-onset diabetes observed in the standard NOD model (23, 24). Our data also suggest that IL-17 may not play a major role in this setting of juvenile-onset diabetes.

To further examine whether IFN-γ plays a role in autoimmune pathogenicity caused by reduced levels of CTLA4, we used anti–IFN-γ Ab treatment. IFN-γ blockade curtailed diabetes development in CTLA4RNAi/B6.H2b7 mice. The treatment suppressed but did not abrogate insulitis in the animals (Fig. 8E–G) nor did it decrease the population size of the CD4+ TEM subset (data not shown). We then examined the role of Th17 by using a line of IL-17A–deficient mice available on the B6 genetic background. As shown in Fig. 1E and 1F, CTLA4 reduction caused insulitis development in B6 mice, although diabetes was not observed. Absence of IL-17A in CTLA4RNAi/B6.IL172mice did not reduce the pancreatic islet infiltration caused by a reduction in CTLA4 (Fig. 9C, 9D) nor did it alter the percentage and number of TEMs in the draining lymph nodes (Fig. 9E, 9F). These results are consistent with the observation that a reduction in the Th17 subset did not lead to a decrease in diabetes development in the CTLA4RNAi/B6.IL172mice treated with IL-7Rα blockade (Fig. 8E, 8F).

Overall, our observations with regard to the Th1 versus the Th17 subsets in autoimmune damage of the pancreatic islets caused by CTLA4 reduction are consistent with the well-recognized pathogenicity of Th1 cells but a debated role of the Th17 subset in autoimmune diabetes (27, 45).

Discussion

Immunological memory is thought to perpetuate chronic damage in autoimmune diseases. Memory cell formation is influenced by a number of peripheral immune regulatory genes, among which CTLA4 levels have long been associated with various autoimmune diseases including T1D (8). It has been shown that CTLA4 blockade increased the population of CD8+CD44hiCD62Llo mice.
T cells, suggesting a role of CTLA4 in CD8+ T cell memory in a model of acute infection (46). However, studies in other acute infection models have shown that CTLA4 does not play an important role in memory formation but rather plays a Treg-mediated role in their quality with respect to cytokine production (47). In our study, we analyzed how a modest variation in CTLA4 levels affects subsets of effector and memory T cells in autoimmune diabetes settings. Multiparametric flow cytometry analyses of the autoimmune models clearly identified T EMs from short-lived TEFFs, both of which are present in the CD44hiCD62Llo pool, with consistent cytokine profiles in ex vivo analyses, and pathogenic potency in vivo as revealed by transfer of autoimmune diabetes.

The novel CTLA4RNAi/B6.H2g7 model recapitulates key aspects of human T1D: onset at juvenile age, natural T cell repertoire, and no gender bias. It offered us an opportunity to examine memory and effector T cell subsets in young animals and to study the effect of a reduction in CTLA4 levels in conjunction with a disease-susceptible MHC. This system also closely resembled human T1D where some disease-susceptible polymorphisms result in lower levels of CTLA4 expression (9–11) and are associated with T1D risk. We also show that although a disease-susceptible MHC is required for progression to frank diabetes onset, a reduction in CTLA4 levels is sufficient to elicit lymphocytic islet infiltration even in a disease-resistant genetic background, as shown in the CTLA4RNAi/B6 model.

In the NOD model, there was an increase of islet-specific glucose-6-phosphatase catalytic subunit related protein–specific CD8+ TEMs in the peripheral lymphoid tissue (spleen and peripheral lymph nodes) after 10 wk of age, which correlated with severity of insulitis in these mice (48). In our CTLA4RNAi/B6 model of juvenile-onset T1D that harbors a natural polyclonal T cell repertoire as in the NOD model, we did not detect substantial impact of CD8+ memory T cells on the early onset of autoimmune diabetes. Although there was also an increase in the total number of short-lived CD4+ TEFFs in the pancreas in this model, the proportion of the CD4+ TEFF compartment (average 2%) was minor compared with the CD4+ T EM subset (average 30%) in the tissue. In addition, our adoptive
CD4+ T cells after anti–IL-7R mononuclear cell (MNC) transfer. IL-7 has been monitored for diabetes for 40 d. (A) Cytometry plots of the pancreas showing IL-17 and IFN-γ production by CD4+ T cells after anti–IL-7R treatment (numbers represent percentage of gated populations). (B and C) Frequencies and total cell numbers of CD4+IL17+ (B) and CD4+IFNγ+ (C) subsets impacted by CTLA4RNAi on the B6.H2b background. Control mice were transgene-negative littermates (n = 4–6/group). (D) Littermate CTLA4RNAi/B6.H2b mice were treated with nonspecific ratIgG2a control Ab or anti–IL-7Rα Ab for 3 wk. Mice were monitored for diabetes for 40 d. (E) Representative intracellular flow cytometry plots of the pancreas showing IL-17 and IFN-γ production by CD4+ T cells after anti–IL-7Rα treatment (numbers represent percentage of gated populations). (F and G) Frequencies and total cell numbers of CD4+IL17+ (F) and CD4+IFNγ+ (G) after anti–IL-7Rα treatment (n = 4–6 mice/group). Each data point represents one animal (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.005.
blocking IL-7 signaling led to reduction in the Th17 population (57). In our CTLA4RNAi model, even though anti–IL-7Rα treatment inhibited differentiation of the Th17 subset, it did not suppress autoimmune Th1 differentiation and did not inhibit the juvenile onset of T1D. A potential role of Th1 effector memory cells appears consistent with a previous study with mouse model of experimental autoimmune encephalomyelitis (58). Therefore, it is possible that failure of the anti–IL-7Rα treatment in curtailing Th1 formation in the young animals may account for the failure of the treatment in suppressing T1D in our model. Indeed, blocking IFN-γ with Ab treatment suppressed diabetes development in the CTLA4RNAi/B6.H2g7 model of juvenile-onset T1D caused by CTLA4 reduction in a natural T cell repertoire. Of note, a fully humanized mAb against IFN-γ has been under clinical trials (ClinicalTrials.gov). Therefore it is possible in the relatively near future to learn whether anti–IFN-γ treatment can suppress juvenile-onset T1D in human beings.

In conclusion, this study of an early-onset model of T1D with a natural T cell repertoire illustrates the apparently opposing effects of CTLA4 insufficiency in enhancing protection by Treg cells but increasing the pathogenicity of the CD4+ Tconv compartment. These dichotomized effects converged at the increased effector memory formation in both Treg and Tconv compartments. Although suppressing memory formation in the Tconv compartment may be desirable in the therapeutic development against autoimmune diseases, promoting the formation of memory Tregs, especially Treg-EMs, could be used to control autoimmune damage. Therefore, although Tregs depend on the presence of CTLA4 for functionality (31), a subtle reduction of CTLA4 levels in Tregs could be engineered to promote Treg-EM formation and thus to promote efficacies of Treg adoptive cell therapies.

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Disclosures
The authors have no financial conflicts of interest.

FIGURE 9. Evidence for IFN-γ but not IL-17 in autoimmune damage to the pancreatic islet caused by CTLA4 downmodulation. (A) Effects of anti–IFN-γ Ab treatment on diabetes incidence in juvenile CTLA4RNAi/B6.H2g7 mice. (B) Summary of islet infiltration in anti–IFN-γ or control Ab–treated mice (n = 4/group). Each bar represents one animal. (C) Representative H&E-stained sections of the pancreas from 6-mo-old mice on the B6 genetic background (original magnification ×12.5). (D) Summary of islet infiltration in 6-mo-old mice on the B6 genetic background. Control mice were CTLA4RNAi-transgene–negative littermates. (E) Flow cytometry analyses of the TEM and Tconv subsets of the CD4+ Tconv compartment in the PLN and pancreas (numbers represent percentages of gated CD4+ Tconv population). (F) Frequencies and total cell numbers of CD4+ TEM subset in IL17+ and IL17− CTLA4RNAi/B6 mice (n = 3/group). Each data point represents one animal (mean ± SEM). *p < 0.05.
CTLA-4 REDUCTION ENHANCES BOTH Treg AND Tconv FUNCTION

References


Supplemental Figure 1. Superior expansion and / or survival of autoantigen-specific CD4+ T_{EM} cells versus CD4+ T_{EFF} cells in a lymphoreplete system. (A) CD4+ T_{EM} cells and CD4+ T_{EFF} cells were purified from BDC2.5/NOD (CD90.2) mice and adoptively transferred into CD90.1 congenic NOD mice. The transferred cells were identified using CD90.2 congenic marker by flow cytometry analyses. The numbers on the gated population are cell numbers calculated per million total cells analyzed for the lymph nodes. (B) Comparison of the total cell numbers of CD90.2+ cells in the PLN of NOD CD90.1 mice transferred with CD4+ T_{EM} versus CD4+ T_{EFF} cells, purified by flow cytometry sorting from BDC2.5 (n=3 from 3 independent experiments). Each data point represents one animal. *p<0.05, ratio-paired t test was used for statistical analysis of the three independent experiments.
Supplemental Figure 2. Anti-CTLA4 antibody treatment increases CD4+ Effector memory formation. (A) Flow cytometry analyses of the naïve, T_{EFF} and T_{EM} subsets of the conventional CD4\(^+\) T cell compartment in the pancreatic lymph nodes (PLN) & pancreas (Numbers represent percentages of gated CD4\(^+\) T_{conv} population). (B-C) Frequencies and total cell numbers of CD4\(^+\) T_{EM} (B) and CD4\(^+\) T_{EFF} (C) cells in anti-CTLA4 treated BDC2.5/NOD mice (n=4-5 per group from 2 experiments). Each data point represents one animal (Mean ±SEM). *p<0.05.
Supplemental Figure 3. Efficacy of anti-IL7Rα treatment. A. Anti-IL7Rα (clone A7R34) treatment resulted in successful blocking of the IL7Rα as indicated by the absence of CD127 staining in the spleen and lymph nodes of treated mice. Numbers represent percentages of gated population. Mice were analyzed by flow cytometry, at 1 day to 5 weeks after treatment regimen was completed (n= 4-5 mice per group). Each data point represents one animal (Mean ± SEM). ***p<0.005.