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Overexpression of the CTLA-4 Isoform Lacking Exons 2 and 3 Causes Autoimmunity

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CTLA-4 is a potent inhibitor of T cell activation, primarily upon binding to its costimulatory ligands (B7.1 and B7.2) expressed on APCs. However, variants of CTLA-4 can also function independently of B7 molecules. I/4CTLA-4 is a highly conserved isoform encoded by exons 1 and 4 of the Ctl4 gene that lacks the ligand-binding and the transmembrane domains, and as yet, its function is not known. To investigate the function of I/4CTLA-4, we generated transgenic (Tg) mice overexpressing this variant. Cytokine production by I/4CTLA-4 Tg T cells was elevated compared with wild type T cells. The frequency of CD44high memory T cells in I/4CTLA-4 Tg mice was increased, and as the mice aged, the frequency further increased. I/4CTLA-4 Tg mice >1 y old had increased expression of T cell activation markers and developed spontaneous autoimmunity, including elevated production of autoantibodies. In contrast with young I/4CTLA-4 Tg mice, aged I/4CTLA-4 Tg mice had elevated frequencies of Foxp3+ regulatory T cells, but the regulatory T cells from these mice were not able to inhibit colitis development. Collectively, these data suggest that the function of the I/4CTLA-4 isoform is distinct from that of CTLA-4 in that it enhances T cell activation and promotes autoimmunity rather than inhibiting immune responses. The Journal of Immunology, 2012, 188: 000–000.

A member of the CD28 family of costimulatory receptors, CTLA-4 binds the same ligands as CD28, namely, B7.1 and B7.2. In contrast with CD28, CTLA-4 is a potent negative regulator of T cell activation, and deletion of Ctl4 has profound effects on peripheral tolerance (1–3). CTLA-4-deficient mice experience development of massive inflammatory infiltrates and tissue damage in multiple organs and exhibit early lethality. Deletion of both B7.1 and B7.2 rescues CTLA-4-deficient mice from lymphoproliferative disease, indicating that hyperactivation of T cells in CTLA-4 KO mice is the result of unchecked CD28/B7 interactions in the absence of inhibitory signal via CTLA-4 (4).

CTLA-4 expression is induced upon T cell activation and constitutively expressed on regulatory T (T-reg) cells (5, 6). CTLA-4 expression on natural T-reg cells is crucial for their suppressive function, because conditional deletion of CTLA-4 in Foxp3+ T-reg cells leads to a breakdown of peripheral tolerance and multorgan tissue inflammation reminiscent of that in CTLA-4 KO (but slower in tempo) and scurfy mice carrying a Foxp3 gene mutation (7, 8). In addition, a recent study showed that silencing of the soluble form of CTLA-4 in T-reg cells impaired their suppressive activity (9). Because of its potent inhibitory effects, CTLA-4 is a target for therapeutic intervention. Ab blockade of CTLA-4 can enhance antitumor immunity, as well as increase cell-mediated immunity, but also has been shown to cause autoimmunity (10–13). An anti–CTLA-4 mAb (ipilimumab) was just approved by the Food and Drug Administration for treatment of human melanomas (14).

Genetic linkage to a locus containing CTLA-4 and ICOS has been reported for multiple autoimmune diseases in mice and humans. These costimulatory receptors are encoded by genes in the type 1 diabetes susceptibility locus on chromosome 1, Idd5.1 (15, 16). In addition to full-length (fl) CTLA-4, three other splice variants have been identified. In humans, the genetic association of CTLA4 with autoimmunity correlates with differential expression of the mRNA encoding the soluble form of CTLA-4, which lacks the transmembrane domain encoded by exon 3, and in nonobese diabetic mice, disease linkage is correlated to the mRNA and protein expression of ligand-independent CTLA-4 (liCTLA-4) (15, 16). liCTLA-4 lacks the ligand-binding Ig domain, and has been reported to be one of the genetic elements that determine susceptibility to diabetes in NOD mice (16). CTLA-4 can also inhibit T cell activation independently of B7 ligation (17, 18), because liCTLA-4 can replace some of the functions of CTLA-4...
and partially rescue CTLA-4–deficient mice from early lethality and lymphoproliferative disease (19). iTcCTLA-4 appears to aid in maintaining self-tolerance; however, this isoform is not expressed in humans. Another isoform of CTLA-4 lacks both the ligand-binding and transmembrane domains encoded by exons 2 and 3, respectively, and is thus named 1/4CTLA-4; this variant is conserved between mice and humans (16). However, the function of 1/4CTLA-4 in the immune system is not known.

To examine the function of 1/4CTLA-4, we generated transgenic (Tg) mice that constitutively overexpress this isoform in T cells. Overexpressing 1/4CTLA-4 in T cells leads to accumulation of activated/memory T cells in the peripheral repertoire and development of autoimmunity. The breakdown in self-tolerance in these mice was associated with hyperactivity of activated/memory T cells together with reduced suppressive activity of Fopx3+ T-reg cells. We provide evidence that, in contrast with the immunosuppressive functions of flCTLA-4 and liCTLA-4, 1/4CTLA-4 enhances T cell responses and induces T cell hyperactivity and spontaneous autoimmunity.

Materials and Methods

Mice and reagents

Thy1.1 congenic and RAG1−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME). Generation of CTLA-4−/−, Fopx3.gfp knock-in (KI) reporter, and 2D2 MOG35-55 TCR Tg mice have been previously described (1, 20, 21). 1/4Ctla4 cDNA was cloned into a pBluescript vector under the control of human CD2 promoter and enhancer regions. Linearized plasmids containing 1/4Ctla4 were injected directly into the pronuclei of fertilized oocytes from C57BL/6 at the Brigham and Women’s Hospital Transgenic Core Facility. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Harvard Medical School (Boston, MA).

Beads for MACS were purchased from Miltenyi Biotec. All flow cytometry reagents were purchased from BioLegend, except CD3–allophycocyanin-Cy7, which was purchased from BD Bioscience. Purified functional-grade Abs for cell culture were obtained from BioXCell.

T cell proliferation assays

Cells were cultured in DMEM containing 10% FCS and supplemented with 1 mM sodium pyruvate, nonessential amino acids, l-glutamine, and 100 U penicillin/100 µg streptomycin per milliliter and 5 × 10−5 M 2-ME. T cell proliferation was assayed using whole spleen and lymph node (LN) cultures stimulated with either soluble anti-CD3 or MOG35-55 peptide, cultures pulsed with 1 µCi/well [3H]thymidine (Perkin Elmer) after 48 h, and [3H]thymidine incorporation was measured on day 3 using a β-counter scintillation counter (1450 Microbeta, Trilux; Perkin Elmer).

Assays using either MACS isolated CD4+ T cells or sorted T cell subsets were cultured in the presence of autologous CD4-depleted splenocytes as APCs, irradiated with 3300 rad. CD4+Fopx3.gfp KI mice were monitored daily and clinical scores were given as follows: 1, limp tail; 2, hind-limb paresis; 3, hind-limb paralysis; 4, tetraplegia; 5, moribund. Brains and spinal cords were harvested for histopathological analysis after 30 d. Pertussis toxin was not given to mice that were sacrificed 8 d postimmunization to investigate cell responses. For spontaneous experimental autoimmune encephalomyelitis (EAE), 1/4CTLA-4 Tg mice were intercrossed with MOG TCR 2D2 Tg mice and scored twice a week; incidence was noted when mice displayed clinical scores ≥1. In addition to brains and spinal cords, eyes and optical nerves were collected for histopathological analysis within a week after clinical signs of EAE were detected.

Colitis disease model

CD4+CD45RBhigh naive T and CD4+GFP T-reg cells were purified by cell sorting after enrichment for CD4+ cells using anti-CD4 MACs beads from Fopx3.gfp KI mice. A total of 2 × 106 CD4+CD45RBhigh cells were transferred into RAG1-deficient mice either alone or cotransferred with T-reg cells (at 1:1 or 1:0.5 effector T cell/T-reg cell ratio) i.p. Mice were weighed weekly after adoptive transfer.

Statistical analysis

Statistical analysis was performed using unpaired, two-tailed Student t tests, and p < 0.05 was considered statistically significant. For spontaneous EAE incidence, log-rank tests were performed using GraphPad Prism.

Results

Distribution and expression kinetics of CTLA-4 variants

CTLA-4 is upregulated upon T cell activation and constitutively expressed by T-reg cells; however, the expression pattern of the 1/4CTLA-4 isoform is not known. By quantitative RT-PCR analysis, we examined the expression pattern of different isoforms of CTLA-4 in T cell subsets ex vivo and after in vitro activation. Remarkably, the expression of the splice variants of CTLA-4 were distributed in a similar pattern to flCTLA-4; that is, very low to undetectable expression in naive T cells, intermediate expression in memory T cells, and most abundantly expressed in T-reg cells (Fig. 1A). Upon activation of T cells with anti-CD3 and anti-CD28, the regulation kinetics of all four CTLA-4 variants appeared biphasic (Fig. 1B). In the cases of flCTLA-4 and liCTLA-4, T cell activation triggered an increase in transcription followed by a second wave of upregulation with a greater magnitude, with the peak of the second phase being at 48 h postactivation. In contrast, soluble CTLA-4 and 1/4CTLA-4 mRNA initially decreased after T cell activation and subsequently increased, also peaking after 48 h postactivation.

CD4+ T cells were isolated by MACs from pooled spleen and LN cells from 2D2 Tg mice and activated with MOG35-55 peptide in the presence of irradiated whole splenocytes from wild type mice. After the cells stopped blasting (usually 3 d), they were rested for 2 d. Cells were washed to remove debris and adoptively transferred i.v. into Thy1.1 congenic mice. Mice were sacrificed after 10 wk, and donor 2D2 Tg cells were identified in the spleen and LNs by staining with CD4 and Thy1.2 Abs for FACS analysis.

Ag arrays

Sera were collected from 1/4CTLA-4 Tg and non-Tg littermates at various ages and hybridized onto glass chips spotted with a panel of self-Ags as previously described (22).

Experimental autoimmune encephalomyelitis disease models

Mice were immunized with MOG35-55 peptide emulsified in CFA (Sigma) containing 4 mg/ml Mycobacterium tuberculosis extract H37Ra (Difco) in 100 µl/mouse s.c. distributed between the two hind flanks and above the sternum on day 0. On days 0 and 2, 100 µg pertussis toxin (List Biological Laboratories) was given i.v. Mice were monitored daily and clinical scores were given as follows: 1, limp tail; 2, hind-limb paresis; 3, hind-limb paralysis; 4, tetraplegia; 5, moribund. Brains and spinal cords were harvested for histopathological analysis after 30 d. Pertussis toxin was not given to mice that were sacrificed 8 d postimmunization to investigate cell responses. For spontaneous experimental autoimmune encephalomyelitis (EAE), 1/4CTLA-4 Tg mice were intercrossed with MOG TCR 2D2 Tg mice and scored twice a week; incidence was noted when mice displayed clinical scores ≥1. In addition to brains and spinal cords, eyes and optical nerves were collected for histopathological analysis within a week after clinical signs of EAE were detected.
exons 2 and 3, raising the issue of whether CTLA-4/A−/− mice still maintained expression of the 1/4CTLA-4 isoform. Thus, we examined the expression of 1/4CTLA-4 in organs of 21-d-old mice. By real-time PCR analysis, soluble CTLA-4 could not be detected in any of the mice (data not shown). As expected, flCTLA-4 and liCTLA-4 were expressed in wild type and CTLA-4+/− littermate samples but were completely undetectable in CTLA-4 KO mice. However, 1/4CTLA-4 expression in the spleen was markedly increased in the KO mice compared with CTLA-4+/+ and CTLA-4 +/− littermates (Fig. 1C). Thymus and heart samples gave the same expression pattern (data not shown). Thus, 1/4CTLA-4 expression was not eliminated in the original CTLA-4 KO mice; rather, its transcript was elevated. This raises the question whether the lymphoproliferative disease and early lethality observed in the CTLA-4−/− is entirely due to loss of flCTLA-4 and liCTLA-4 or whether overexpression of the 1/4CTLA-4 isoform contributes to this disease phenotype.

Overexpression of 1/4CTLA-4 leads to elevated cytokine production in T cells

To investigate the role of 1/4CTLA-4 in vivo, Tg mice overexpressing this genetic variant were generated on a wild type C57BL/6 background (Fig. 2). Because gene expression in these mice is under the control of the human CD2 promoter, 1/4CTLA-4 Tg mice overexpress 1/4Ctla4 in T cells. In these mice, T cells, either ex vivo or activated in vitro, overexpress 1/4CTLA-4, as confirmed by real-time PCR (Fig. 2B). Three different 1/4CTLA-4 Tg lines were generated and all of the lines displayed a similar phenotype; most of the data presented in this article are from the 2167 founder line.

To investigate T cell function in 1/4CTLA-4 Tg mice, we stimulated cells from peripheral LN and spleen in vitro. Thymidine incorporation assays showed that proliferation of cells from naive 1/4CTLA-4 Tg mice was not consistently different from cells of wild type littersmates upon activation with soluble anti-CD3; however, cytokine production by T cells was always elevated in 1/4CTLA-4 Tg mice (Fig. 3A). By ELISA, we found that IL-2, IL-17, IFN-γ, TNF, IL-10, and IL-6 production were all higher in T cells from 1/4CTLA-4 Tg mice compared with T cells from non-Tg littermates. IL-4 and IL-5 production were below the limits of detection (data not shown). Thus, overexpression of 1/4CTLA-4 leads to general elevation of cytokine production not biased toward any particular T helper subset.

Highly purified naive (Foxp3/GFP−CD44lowCD62Lhigh) and memory (Foxp3/GFP−CD44high) CD4+ T cells were further examined to investigate the effect of overexpression of 1/4CTLA-4 on proliferation of these subsets. Upon activation with anti-CD3 in vitro, in the presence of irradiated syngeneic APCs, memory T cells from 1/4CTLA-4 Tg mice proliferated more than their wild type counterparts (Fig. 3B). Naive T cells from Tg and non-Tg animals, in contrast, proliferated similarly. In CD4+Foxp3/GFP− cells that were not further fractionated, proliferation was similar to naive T cells, likely because of the fact that only a small fraction of the Foxp3−/GFP− cells were activated/memory cells.

To investigate the fate of 1/4CTLA-4 Tg activated/memory T cells in vivo, 1/4CTLA-4 Tg mice were crossed with MOG 35–55 TCR2D2 Tg Thy1.2 mice. T cells from 2D2 Tg Thy1.2 mice were activated in vitro with MOG 35–55 peptide, allowed to rest (5 d in total), and then parked in Thy1.1 congenic mice. After 10 wk, most of the donor cells were recovered in the spleen with significantly more 1/4CTLA-4 Tg donor cells recovered compared with non-Tg donor cells (p = 0.0069; Fig. 3C), further demonstrating in...
Activated/memory T cells accumulate in 1/4CTLA-4 Tg mice

Because activated/memory, and not naive, T cells from 1/4CTLA-4 Tg mice show increased proliferative responses, we set out to determine whether this hyperproliferation would affect the composition of T cells in 1/4CTLA-4 Tg mice. Examination of the lymphoid compartments showed no defect in thymic development (Supplemental Fig. 1). The proportions of T and B cells in the spleen and peripheral LNs were similar to those in non-Tg littermates (Supplemental Fig. 1). Up to 10 wk of age, the cellularity and expression of activation markers (CD25, CD69) on T cells from the lymphoid organs were no different between non-Tg and 1/4CTLA-4 Tg littermates (Fig. 4A, Supplemental Fig. 1). However, 1/4CTLA-4 Tg mice had significantly increased frequencies of CD4+ memory (CD44high) T cells in the spleen and showed a trend toward increased frequency of memory T cells in both the CD4 and CD8 compartments in the peripheral lymphoid compartments (Fig. 4B).

Interestingly, the accumulation of activated/memory T cells observed in 1/4CTLA-4 Tg mice was more obvious as the mice aged. In mice older than 1 y, the frequencies of T cells expressing CD25 and CD69 were elevated in 1/4CTLA-4 Tg mice compared with wild type littermates (Fig. 4C, Supplemental Fig. 1C). It should be noted that in some of the 1/4CTLA-4 Tg mice, almost all CD4+ (>80%) and CD8+ (>75%) T cells in the periphery expressed high CD44 at high levels (Fig. 4C). Further, the accumulation of CD4+ memory T cells was so great that the frequency of naive T cells remaining in the spleen and LNs in some mice was very low (Fig. 4C) and was evident in both the CD4+ and CD8+ compartments. However, the proportions of CD4+ and CD8+ T cells in the secondary lymphoid organs were not altered by overexpression of 1/4CTLA-4, even with increased age (Supplemental Fig. 1B, 1C). With the increased frequency of activated/memory T cells in the peripheral repertoire, some, but not all, mice >1 y old experienced development of lymphadenopathy and splenomegaly (Fig. 4D). Upon histological analyses, the major organs in 1/4CTLA-4 Tg mice were grossly normal, and in mice older than 1 y, there were no signs of inflammatory infiltrates in contrast with the inflammation that develops in multiple organs in the CTLA-4 KO mice (data not shown).

Increased accumulation of activated/memory T cells can result from increased proliferation or increased survival, or both. We showed that CD44high T cells were hyperproliferative (Fig. 3B, 3C); thus, in vitro activation-induced cell death assays were performed to assess T cell survival. 1/4CTLA-4 Tg T cells in general did not survive better than T cells from non-Tg littermates upon restimulation (Supplemental Fig. 2). Our data thus indicate that activated/memory T cells accumulate in 1/4CTLA-4 Tg mice as the mice age because of hyperproliferation of the activated/memory T cell subset.

Autoantibody responses in mice overexpressing 1/4CTLA-4

Because T cells from naive 1/4CTLA-4 Tg mice accumulate activated/memory T cells, we investigated development of autoimmunity in these mice. First, we assessed the production of autoantibodies in 1/4CTLA-4 Tg mice using Ag arrays spotted with a panel of self-Ags. We found that when compared with wild type littermates, 1/4CTLA-4 Tg mice had elevated autoantibody levels in their sera that reacted toward a wide range of Ags associated with diverse autoimmune diseases, rather than specific to any one autoimmune disease (Fig. 5A). This suggests that the self-reactivity was not restricted to a particular tissue or organ. In line with the increased accumulation of activated/memory T cells in older 1/4CTLA-4 Tg mice, the production of autoantibodies strongly correlated with the age of the mice (Fig. 5B).

Given that overexpression of 1/4CTLA-4 in these mice is restricted to T cells, the effect of overexpression of this gene on elevated autoantibody production is likely due to T-dependent B cell responses. T follicular helper cells have been described as having a critical role in the generation of germinal centers and Abs (23, 24); therefore, we examined the generation of this subset in 1/4CTLA-4 Tg mice. Mice were immunized with MOG35-55 peptide emulsified in CFA and the frequency of CD4+ CXCR5+ICOS+ cells in the draining LNs and spleens were determined 6 d later. There was no apparent difference in the generation of T follicular helper cells in 1/4CTLA-4 Tg and non-Tg mice upon immunization (Supplemental Fig. 3A). It is conceivable that immunization with a high dose Ag together with adjuvant can
mask any subtle effects that overexpression of 1/4CTLA-4 could have on the generation of T follicular helper cells. Thus, we examined generation of germinal centers in aged 1/4CTLA-4 mice without immunization. 1/4CTLA-4 Tg mice aged to 1 y developed germinal centers spontaneously containing a distinct population of germinal center B cells (CD19⁺PNA⁺Fas⁺GL7⁺IgD⁺) that was minor in the peripheral LN and scarce in the spleen of non-Tg littermates (Supplemental Fig. 3B). Thus, there is a difference in help provided to B cells in 1/4CTLA-4 Tg mice as the mice aged.

Autoimmunity and Ag-specific responses in mice overexpressing 1/4CTLA-4

To investigate the role of 1/4CTLA-4 in autoimmunity, we assessed Ag-specific T cell responses. Mice were immunized with an epitope of myelin Ag MOG 35–55 peptide emulsified in CFA, and the cells from draining LNs were restimulated in vitro 8 d later to examine Ag-specific recall responses. T cells from 1/4CTLA-4 Tg mice proliferated more and produced more IL-17 when restimulated in vitro with MOG 35–55 peptide than T cells from wild type littermates (Fig. 6A). Given the elevated Ag-specific responses of T cells from 1/4CTLA-4 Tg mice observed in vitro, we next examined the role of this variant form of CTLA-4 in T cells using different models of EAE. After immunization with a suboptimal dose (50 μg) of MOG35–55/CFA plus pertussis toxin, 1/4CTLA-4 Tg mice experienced development of more severe EAE than wild type littermate controls (Fig. 6B). The incidence of clinical and histopathological disease was higher and the onset of disease was accelerated in 1/4CTLA-4 Tg mice. Of the wild type mice that did experience development of EAE, the mean maximal clinical score was lower than in 1/4CTLA-4 Tg mice (2.3 ± 0.837 versus 3 ± 0, mean ± SD). However, immunization with a higher dose of MOG35–55 peptide (100 μg) yielded similar incidence and severity in the littermate controls as the mice overexpressing 1/4CTLA-4 (data not shown), suggesting that strong immunization regimens can override differences caused by the 1/4CTLA-4 transgene. Therefore, 1/4CTLA-4 Tg mice crossed with MOG35–55-specific 2D2 TCR Tg mice were used to investigate the development of EAE in the absence of immunization. None of the 2D2 TCR Tg mice in our study experienced development of EAE, but 40% of 1/4CTLA-4 Tg mice on the 2D2 Tg background experienced development of EAE spontaneously, with clinical signs of disease beginning as early as 4 wk of age (Fig. 6C). Thus, 1/4CTLA-4 Tg mice have increased Ag-specific T cell responses and develop more severe EAE than wild type littermates, but this difference can be overridden by strong immunization regimens. Results from the two different models of EAE and the Ag arrays together suggest that overexpression of 1/4CTLA-4 leads to autoimmunity.
Given that 1/4CTLA-4 is expressed constitutively at very high levels in T-reg cells (Fig. 1), and mice overexpressing this isoform develop autoimmunity (Figs. 5, 6), we set out to determine whether overexpression of 1/4CTLA-4 had an effect on T-reg cell development. In the younger cohort of Foxp3.gfp KI reporter mice (8–10 wk old), the frequency of CD4+Foxp3/GFP+ T-reg cells in the periphery was not different in 1/4CTLA-4 Tg mice and wild type littersmtes (Fig. 7A, Supplemental Fig. 1B, 1C). In aged mice, T-reg cell frequencies were slightly elevated in 1/4CTLA-4 Tg mice compared with non-Tg littersmates. That is, spleens of aged 1/4CTLA-4 Tg mice (>1 y old) had elevated T-reg cell frequencies in the CD4 compartment compared with littersmate non-Tg mice (p = 0.0094); in the peripheral LNs, there was only a trend toward increased T-reg cell frequencies (Fig. 7A, Supplemental Fig. 1C). Accumulation of T-reg cells in aged mice may reflect either an indirect effect because of the increase in the pool of activated/memory T cells, or a direct effect of overexpressing 1/4CTLA-4 in T-reg cells. The suppressive activity of highly purified CD4+Foxp3/GFP+ T-reg cells from 1/4CTLA-4 Tg and non-Tg littersmates was compared using in vitro suppression assays and no clear difference was observed (data not shown). Thus, we investigated the suppressive ability of T-reg cells in vivo using a model of colitis. When naive T cells (CD4+CD45RB$^{\text{high}}$) from wild type mice were cotransferred with T-reg cells at 1:0.5 ratio, RAG1$^{-/-}$ mice cotransferred with wild type T-reg cells were protected from colitis, whereas mice receiving 1/4CTLA-4 Tg T-reg cells experienced development of colitis similar to mice receiving only naive T cells (Fig. 7B). When more T-reg cells were cotransferred, at a 1:1 ratio, all mice were protected from colitis (data not shown). Neither 1/4CTLA-4 Tg nor non-Tg T cells on their own caused weight loss in the RAG2$^{-/-}$ recipient mice (Supplemental Fig. 4). These data suggest that T-reg cells from 1/4CTLA-4 Tg mice were less efficient at suppressing the effector functions of cotransferred naive T cells. Of note, weight loss in recipient RAG1$^{-/-}$ mice was similar between mice reconstituted with donor 1/4CTLA-4 Tg- and non-Tg-derived naive T cells (Supplemental Fig. 4), further demonstrating that naive T cells from 1/4CTLA-4 Tg mice were not hyperproliferative.

**Discussion**

It is well established that flCTLA-4 is a potent negative regulator of T cell activation. We previously demonstrated that liCTLA-4, an isoform associated with type 1 diabetes in mice, also inhibits T cell activation and replaces some of the functions of flCTLA-4 in mice (17, 19). Whereas liCTLA-4 is not expressed in humans, 1/4CTLA-4 is present in humans and mice, and also lacks the ligand-binding domain. To investigate the function of 1/4CTLA-4 in T cells, we generated Tg mice in which 1/4CTLA-4 is overexpressed constitutively and specifically in T cells. Upon stimulation, T cells from 1/4CTLA-4 Tg mice produce more cytokines than their wild type counterparts, most likely as a result of increased accumulation of activated/memory T cells in peripheral lymphoid tissues. As these mice age, this phenotype becomes more dramatic. Together with defective T-reg cell function, this culminates in autoimmunity in 1/4CTLA-4 Tg mice.

It has previously been shown that CTLA-4−deficient mice display early lethality caused by aggressive infiltration and damage of multiple organs by inflammatory cells and have hyperactive T cells (1, 2). However, our studies reveal that these mice lack all other isoforms of CTLA-4 (flCTLA-4, soluble CTLA-4, and liCTLA-4), but overexpress 1/4CTLA-4 in the periphery (Fig. 1C). When 1/4CTLA-4 was overexpressed in CTLA-4$^{-/-}$ mice, a similar, but milder, form of the phenotype developed, raising the issue of how much overexpression of the 1/4CTLA-4 isoform contributed to the T cell hyperactivity observed in CTLA-4−deficient mice. These 1/4CTLA-4 Tg mice progressively accumulated activated/memory T cells and produced autoantibodies against many self-Ags (Fig. 5), but gross lymphadenopathy and splenomegaly was observed in only a small cohort of the Tg mice. However, spontaneous disease was not evident until mice were crossed with self-Ag TCR-restricted Tg mice (Fig. 6C). Similar to CTLA-4 KO mice,
1/4CTLA-4 Tg mice had elevated Ab production to a diverse array of self-Ags (2). As Tg expression of 1/4CTLA-4 in our mice is under the control of the human CD2 promoter, overexpression is restricted to T cells. Therefore, elevated autoantibody production in these mice (Fig. 5) likely reflects an increase in T-dependent B cell help (23, 24).

Our data suggest that the T cell subsets directly affected by overexpression of 1/4CTLA-4 are the subsets that usually express the CTLA-4 variants. Although Tg overexpression of 1/4CTLA-4 in our mice was on all T cells, it was the activated/memory cells, and not the naive T cells, that displayed altered T cell proliferation compared with their non-Tg counterparts (Fig. 3, Supplemental Fig. 4). Indeed, elevated cytokine production detected in the cultures containing bulk splenocytes and LN cells from 1/4CTLA-4 Tg mice is consistent with increased accumulation of activated/memory T cells (Fig. 3A). As with other splice variants, 1/4CTLA-4 is induced in activated T cells and constitutively and abundantly expressed in T-reg cells (Fig. 1). However, the proportion of CD4+ T cells that were Foxp3+ remained unaltered by Tg overexpression until the mice were older (Fig. 7). Overexpression of 1/4CTLA-4 resulted in T-reg cells that were less efficient in protecting mice from colitis in a T cell transfer model. Indeed, blockade of CTLA-4 in a similar model of colitis abrogated the suppressive activity of T-reg cells (13). By specifically deleting CTLA-4 on T-reg cells, it was recently shown that CTLA-4 expression is essential for T-reg cell function. Mice with CTLA-4–deficient T-reg cells exhibited a less aggressive form of the multiorgan lymphoproliferative disorder of CTLA-4 null mice, suggesting that CTLA-4 in both effector and T-reg cells contribute to maintaining peripheral tolerance (7). Whether overexpression of 1/4CTLA-4 in the Tg mice enhances functions of effector T cells or interferes with the functions of T-reg cells, or whether loss of T-reg cell function results in the observed phenotype remains to be seen. Further investigation will be required to elucidate the mechanisms of how the 1/4CTLA-4 splice variant enhances T cell activation. Both soluble CTLA-4 and liCTLA-4 have previously been reported to be functional variants despite the absence of transmembrane and ligand-binding domains, respectively. It is conceivable that 1/4CTLA-4 could act as a dominant negative or alter the assembly of CD28 versus liCTLA-4 with the TCR in lipid rafts, thus stabilizing the immune synapse and promoting TCR signaling. 1/4CTLA-4 is a small transcript composed of 166 bp (16); with the lack of specific reagents available, it is not known whether 1/4CTLA-4 mRNA is even translated as a protein. It is possible that 1/4CTLA-4 could act directly at the mRNA level to inhibit the expression of other isoforms of CTLA-4 like a short hairpin RNA or microRNA.

In this study, we show that 1/4CTLA-4 overexpressed in T cells affects the function of activated/memory T and T-reg cells. Our
data indicate that, unlike liCTLA-4, 1/4CTLA-4 has distinct and likely opposing functions to fCTLA-4. Therefore, isoform-specific manipulation of 1/4CTLA-4 expression represents a new avenue for therapeutic intervention for inflammatory diseases and tumor progression.

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Disclosures
The authors have no financial conflicts of interest.

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