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Re-establishing Peripheral Tolerance in the Absence of CTLA-4: Complementation by Wild-Type T Cells Points to an Indirect Role for CTLA-4

Elizabeth A. Tivol and Jack Gorski

CTLA-4 plays an important role in the down-regulation of activated T cells and in the establishment of peripheral tolerance. It has been hypothesized that CTLA-4 on the cell surface signals directly into T cells during primary immune responses, resulting in intrinsic T cell down-regulation. It is not known, however, whether CTLA-4 directly inhibits the less intense activating signals received by autoreactive T cells in the periphery. We investigated whether CTLA-4 acts intrinsically upon self-reactive cells in vivo, or whether it inhibits autoreactive cells indirectly, in a non-cell autonomous manner. The adoptive transfer of CTLA-4-deficient splenocytes or Thy 1+ cells into recombinase-activating gene 2-deficient mice resulted in fatal inflammation and tissue destruction similar to that seen in CTLA-4-deficient mice. When an equivalent number of splenocytes or Thy 1+ cells from wild-type animals was transferred with the CTLA-4-deficient cells, recipient mice survived indefinitely. Since CTLA-4 was absent in the T cells responsible for the inflammatory phenotype, the down-regulation of these autoreactive cells must have been facilitated indirectly by wild-type Thy 1+ cells. In addition, a rapid reduction in the ratio of CTLA-4-deficient to wild-type cells was observed. We propose two possible indirect mechanisms by which CTLA-4 may function in the establishment and maintenance of peripheral tolerance. The Journal of Immunology, 2002, 169: 1852–1858.

During thymic selection, thymocytes that react too strongly to self Ags fail to develop into mature T cells (1). Some autoreactive T cells, however, escape negative selection and migrate into the periphery. Normally these cells do not participate in destructive immune reactions against self due to mechanisms of peripheral tolerance, such as that mediated by Fas and Fas ligand. When peripheral tolerance fails, as in Fas-deficient individuals, self-reactive T cells become activated, and disease results.

The protein CTLA-4 has been shown to attenuate activated T cells and their responses (2–5). In addition, CTLA-4 function is required for the establishment of peripheral tolerance in vivo (6, 7). The importance of CTLA-4 in the induction of peripheral tolerance is best demonstrated by CTLA-4-deficient mice (8, 9). Self-reactive T cells in these mice cause lymphoproliferative disease accompanied by fatal tissue destruction in a variety of organs. CTLA-4-deficient mice have normal thymic development (10, 11), suggesting that the disease results from failure of peripheral tolerance of T cells.

The cytoplasmic tail of CTLA-4 has been shown to be associated with proteins involved in intracellular signaling, including phosphatidylinositol 3-kinase, Src homology domain containing tyrosine phosphatase 2, Src kinases, PP2A, and the CD3 δ-chain (12–17). In addition, cross-linking of CTLA-4 on the surface of T cells has been shown to cause their down-regulation or deletion (18, 19). These data suggest that CTLA-4 acts directly within activated T cells by transmitting an interfering, competing, or inhibitory signal directly into the T cell, resulting in its down-regulation. While CTLA-4 may directly down-regulate activated T cells in a primary costimulation-dependent immune response, it is not clear in which population of T cells that CTLA-4 may be acting to achieve peripheral tolerance, nor is the relationship clear between the strength of the activation signal and CTLA-4 function. Most studies of CTLA-4 signaling and function have used conditions that maximize T cell activation. Self-reactive T cells in the periphery, however, frequently encounter autoantigens under suboptimal conditions, such as in the absence of inflammatory cytokines and costimulatory molecules. Under these circumstances, CTLA-4 may not down-regulate T cells to the same extent or in the same manner.

Bone marrow transplant studies by Bachmann et al. (20) have shown that cells that express CTLA-4 are capable of inhibiting the development or function of autoreactive CTLA-4-deficient cells. In addition, CTLA-4-deficient T cells specific for exogenous Ags respond normally in vivo when challenged in the presence of wild-type T cells (21). These data indicate that at least some of the functions of CTLA-4 are not T cell autonomous. In the studies performed here we use an adoptive transfer approach to expand upon these observations and explore whether CTLA-4 inhibits autoreactive peripheral T cells by a T cell autonomous mechanism.

Materials and Methods

Mice

CTLA-4-deficient mice on the 129 background were generated and genotyped as previously described (8). Recombinase-activating gene 2 (RAG-2)-deficient mice on the 129 background were obtained from Taconic Farms ( Germantown, NY). Adoptive transfer recipients were injected i.v. with up to 24 × 10⁶ splenocytes or 8 × 10⁶ Thy 1.2+ T cells. Mice injected with a mixture of cells received the maximal number of cells indicated

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3 Abbreviations used in this paper: RAG-2, recombinase-activating gene 2.
(2 × 10^6 or 8 × 10^5). Mice given only wild-type or CTLA-4-deficient cells received either the same or half (12 × 10^6 or 4 × 10^5) the maximal number of cells with no difference in phenotype.

**Cell preparations**

CTLA-4-deficient T cells were isolated from mice at 16 days of age. Cell suspensions were prepared by grinding tissue through sterile wire mesh. Thy 1.2^+ cells were isolated using CD90 magnetic MicroBeads from Miltenyi Biotec (Auburn, CA) in accordance with the manufacturer’s instructions. Cells used in adoptive transfer experiments were washed into HBSS and injected i.v.

**Cell surface staining**

Cells were stained with a panel of fluorochrome-conjugated Abs, including FITC-conjugated, PE-conjugated, and Tri-Color-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-B220. Abs were purchased from Caltag (Burlingame, CA) or BD PharMingen (San Diego, CA). Stained cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA).

**Histological analysis**

Tissue for light microscopy was fixed in 10% buffered formalin, processed, and embedded in paraffin. Sections were stained with H&E using standard techniques.

**Determination of wild-type/CTLA-4-deficient cell ratios**

Spleen and lymph nodes were taken from adoptive transfer recipients, and T cells were purified using CD90 magnetic MicroBeads from Miltenyi Biotec in accordance with the manufacturer’s instructions. DNA was extracted from T cells using standard techniques and was used as a substrate for PCR. Both the wild-type and disrupted forms of the CTLA-4 gene were amplified in a single reaction using an antisense primer labeled with FAM for PCR. Both the wild-type and disrupted forms of the CTLA-4 gene were amplified in a single reaction using an antisense primer labeled with FAM. PCR products were electrophoresed on a 5% acrylamide gel, and the fluorescence intensities of the bands representing the wild-type and disrupted CTLA-4 alleles were quantitated using a FluorImager 575 (Molecular Dynamics, Sunnyvale, CA) and ImageQuant and spreadsheet software.

Relative intensities were corrected for primer efficiency and for the efficiency of CD90^-^ T cell purification. Primer efficiency was calculated by amplifying the CTLA-4 alleles in tail DNA prepared from mice heterozygous for the disrupted CTLA-4 gene. Heterozygous mice necessarily carry 50% wild-type and 50% CTLA-4-deficient alleles in all tissues, and primer efficiencies were normalized on this basis. The background signal of the gel was defined as the intensity obtained at the band position of the disrupted CTLA-4 allele in mice that received only wild-type splenocytes. This number was subtracted from the intensities of all the bands representing the disrupted CTLA-4 allele. The intensity of the wild-type band in mice that received only CTLA-4-deficient splenocytes was used to estimate the contribution of cells from the recipient RAG-2-deficient animals in the T cell preparations. The fractional intensity of this band was multiplied by the total of the band intensities for each mouse and subtracted from the intensities of the bands representing the wild-type CTLA-4 allele.

**Rearrangement analysis of TCR DNA**

Rearrangement analysis was performed by PCR amplification of the CDR3 using Vβ and Jβ region-specific primers as previously described (22, 23). The Vβ6.2 primer was unlabeled, and the Jβ2.1 region primer was labeled with FAM. PCR products were electrophoresed on a 5% acrylamide gel, and the fluorescence intensities of the bands were quantitated using a FluorImager 575 (Molecular Dynamics) and ImageQuant and spreadsheet software. The relative intensity of each band was calculated by dividing its intensity by the sum of the intensities of the indicated major bands.

**Results**

CTLA-4-deficient mice develop a spontaneous lymphoproliferative disease with inflammation and multorgan tissue destruction. The disease proves fatal at ~3 wk of age on an inbred 129 background. Tissue destruction is Ag specific, since CTLA-4-deficient, TCR transgenic mice on a RAG-deficient background do not develop disease (11, 24–27). Development of the CTLA-4-deficient phenotype additionally requires CD28 engagement (28, 29). In the most frequently proposed mechanism of CTLA-4 function, CTLA-4 on the cell surface intrinsically inhibits T cells activated by Ag and CD28. According to this model, CTLA-4-deficient mice develop autoimmune disease because peripheral T cells activated by self Ags cannot express CTLA-4 and thus cannot be down-regulated.

We used an adoptive transfer approach to investigate the validity of this model. Transferring splenocytes from CTLA-4-deficient mice into RAG-2-deficient mice, which possess no endogenous T or B cells, resulted in a fatal lymphoproliferative disease similar to that of CTLA-4-deficient mice (Fig. 1). The adoptive transfer of 24 × 10^6 CTLA-4-deficient splenocytes caused death in an average of 36 days, and 12 × 10^6 cells in an average of 44 days. To develop inflammatory disease by CTLA-4-deficient splenocytes. RAG-2-deficient mice were injected i.v. on day 0 with the indicated number (×10^6), and ratio of splenocytes indicated in the inset. a and b, All CTLA-4-deficient splenocytes (n = 10 and 9); c, 75:25 ratio of CTLA-4-deficient to wild-type splenocytes (n = 10); d, 50:50 ratio of CTLA-4-deficient to wild-type splenocytes (n = 9); e, all wild-type splenocytes (n = 9). Numbers (n) represent the total number of animals receiving cells in nine separate adoptive transfer experiments.

FIGURE 1. Wild-type splenocytes enhance the survival of mice receiving CTLA-4-deficient splenocytes. RAG-2-deficient mice were injected i.v. on day 0 with the indicated number (×10^6), and ratio of splenocytes indicated in the inset. a and b, All CTLA-4-deficient splenocytes (n = 10 and 9); c, 75:25 ratio of CTLA-4-deficient to wild-type splenocytes (n = 10); d, 50:50 ratio of CTLA-4-deficient to wild-type splenocytes (n = 9); e, all wild-type splenocytes (n = 9). Numbers (n) represent the total number of animals receiving cells in nine separate adoptive transfer experiments.

FIGURE 2. The presence of wild-type splenocytes inhibits the development of inflammatory disease by CTLA-4-deficient splenocytes. H&E-stained sections from pancreas (A, C, and E) and liver (B, D, and F) from RAG-2-deficient mice that received 24 × 10^6 wild-type splenocytes (A and B), CTLA-4-deficient splenocytes (C and D), or a 50:50 mixture of wild-type and CTLA-4-deficient splenocytes (E and F) are shown. Tissues were harvested 29 days (wild-type or CTLA-4-deficient splenocytes alone) or 62 days (combination of wild-type and CTLA-4-deficient splenocytes) after adoptive transfer.
determine whether disease resulted from the lack of direct inhibition by CTLA-4 on the autoreactive T cells, a mixture of wild-type and CTLA-4-deficient splenocytes was adoptively transferred. If the disease results from the inability of autoreactive T cells to be directly inhibited by CTLA-4 on the cell surface, the presence of additional wild-type cells should have no effect on disease development. However, if CTLA-4 exerts its down-regulatory effects via a more complex mechanism, such as through a population of regulatory cells, the presence of wild-type cells may alter the course of the disease. The adoptive transfer of a 25:75 mixture of wild-type to CTLA-4-deficient splenocytes resulted in an increase in the rate of survival of recipient animals. Mice receiving a 50:50 mixture of wild-type to CTLA-4-deficient splenocytes survived indefinitely with no outward signs of disease (n = 9), as did mice receiving wild-type cells alone (n = 9). Thus, CTLA-4-deficient splenocytes are capable of causing fatal lymphoproliferative disease, and wild-type splenocytes are capable of inhibiting disease-related mortality.

Histological analysis of tissues from the RAG-2 recipients indicates that survival time reflects the presence or lack of tissue infiltration and destruction. Mice that were given wild-type splenocytes alone showed no histological evidence of inflammation or tissue destruction in any of the tissues examined (Fig. 2, A and B). RAG-2-deficient mice that received splenocytes from CTLA-4-deficient mice, however, exhibited inflammation and tissue destruction similar in distribution and severity to that of CTLA-4-deficient mice (Fig. 2, C and D). Affected tissues included the pancreas, skeletal muscle, heart, liver, and lung. Organs that are normally unaffected in CTLA-4-deficient mice were unaffected in adoptively transferred animals as well. Adoptively transferring wild-type splenocytes with the CTLA-4-deficient splenocytes not only enhanced survival, but also inhibited the development of inflammation and tissue destruction. Animals receiving a 50:50 mixture of wild-type and CTLA-4-deficient splenocytes showed little or no histological evidence of inflammation in any of the tissues normally affected in CTLA-4-deficient mice (Fig. 2, E and F). The ability of wild-type cells to inhibit the development of disease by CTLA-4-deficient cells implies that the role of CTLA-4 in peripheral tolerance is mediated via an indirect, nonintrinsic mechanism on the autoreactive cells.

To identify the cells involved in the development and amelioration of disease, purified Thy 1.2+ cells were adoptively transferred. Peripheral Thy 1.2+ cells from CTLA-4-deficient mice were sufficient to transfer the fatal lymphoproliferative disease to RAG-2-deficient mice (Fig. 3). Cotransferring wild-type Thy 1.2+ cells and CTLA-4-deficient Thy 1.2+ cells resulted in enhanced survival of the recipient animals, as was seen in mice receiving splenocyte mixes. Mice receiving a 50:50 mixture of Thy 1.2+ T cells survived as well as mice receiving wild-type Thy 1.2+ T cells alone. Histological analysis indicated that the life span of the recipient animals was a reflection of the degree of inflammation and tissue destruction. Mice that received CTLA-4-deficient Thy 1.2+ cells alone exhibited fulminant infiltrates and tissue destruction, whereas mice that received a 50:50 mixture of wild-type and CTLA-4-deficient Thy 1.2+ cells both survived and showed no histological evidence of disease (Fig. 4). Thus, CTLA-4-deficient T cells alone are capable of transferring fatal autoimmune disease to RAG-2-deficient mice, and wild-type T cells are capable of inhibiting disease development.

FIGURE 3. Wild-type T cells enhance the survival of mice receiving CTLA-4-deficient T cells. RAG-2-deficient mice were injected i.v. on day 0 with Thy 1.2+ T cells of the indicated type and ratio. A, All CTLA-4-deficient T cells (n = 7); 50:50 ratio of CTLA-4-deficient to wild-type T cells (n = 6). Data represent mice receiving cells in four different adoptive transfer experiments.

FIGURE 4. The presence of wild-type T cells inhibits the development of inflammatory disease by CTLA-4-deficient T cells. H&E-stained sections from pancreas, liver, and skeletal muscle from RAG-2-deficient mice that received 8 x 10^6 wild-type Thy 1.2+ T cells (WT), CTLA-4-deficient Thy 1.2+ T cells (KO), or a 50:50 mixture of wild-type and CTLA-4-deficient Thy 1.2+ T cells (MIX) are shown. Tissues were harvested 30 days after T cells were adoptively transferred.
We investigated the fate of the CTLA-4-deficient cells in these animals to determine whether they were tolerized by the wild-type cells or were eliminated. To this end, we examined the relative ratio of wild-type to CTLA-4-deficient T cells in Thy 1.2+ splenocytes from adoptive transfer recipients using the presence of wild-type or disrupted CTLA-4 alleles as markers (Fig. 5). While the splenocytes initially injected into the RAG-2-deficient recipients contained a 50:50 ratio of wild-type to CTLA-4-deficient cells, the fraction of CTLA-4-deficient Thy 1.2+ cells was reduced within 2 days of transfer. It is unlikely that CTLA-4-deficient cells selectively died during the adoptive transfer process, as functional CTLA-4-deficient T cells survive the adoptive transfer process well when transferred alone. In fact, after 25 days mice receiving only CTLA-4-deficient cells had ~10 times as many lymphocytes in the periphery as were initially transferred, whereas mice receiving only wild-type cells had only doubled their cell number. Mice receiving a 50:50 mixture of wild-type and CTLA-4-deficient cells had approximately the same number or fewer lymphocytes as mice receiving wild-type cells alone (data not shown). These data suggest that the wild-type cells in mice receiving mixed splenocytes rapidly and selectively eliminated many of the CTLA-4-deficient cells.

To look more specifically at the CTLA-4-deficient T cells in the adoptive transfer recipients, we took advantage of the fact that the expansion of CTLA-4-deficient T cells in the periphery is oligoclonal. By analyzing the spectrum of TCR CDR3 lengths in the DNA from the original CTLA-4-deficient T cells, some expanded sets of T cells were identified as strong intensity bands. These expansions were then followed in the splenic DNA of adoptive transfer recipients (Fig. 6). Thy 1.2+ cells were purified from adoptive transfer recipients, and DNA encoding the CDR3 region of the TCR β-chains was amplified using Vβ- and Jβ-specific primers. The profiles for Vβ8.2 to Jβ2.1 rearrangements are shown, as they represent an informative example. The wild-type

**FIGURE 5.** The ratio of CTLA-4-deficient T cells to wild-type T cells rapidly decreases upon adoptive transfer. Following the adoptive transfer of a 50:50 mixture of wild-type and CTLA-4-deficient splenocytes, the relative frequencies of wild-type and CTLA-4-deficient Thy 1.2+ splenocytes (A) or LN cells (B) were analyzed over time.

**FIGURE 6.** Adoptively transferred CTLA-4-deficient T cells are eliminated over time. Following the adoptive transfer of a 50:50 mixture of wild-type and CTLA-4-deficient splenocytes, mice were sacrificed at the indicated times, and DNA was prepared from CD90+ splenocytes. The DNA was amplified using primers specific for Vβ8.2 and Jβ2.1, and the PCR products were electrophoresed. A, The PCR products resulting from the amplification of DNA from starting wild-type splenocytes, CTLA-4-deficient splenocytes, and the 50:50 mixture of splenocytes injected on day 0 are compared with those obtained at the indicated numbers of days posttransfer. Major bands resulting from in-frame rearrangements of the TCR β-chain locus are numbered. An out-of-frame rearrangement in T cells derived from a CTLA-4-deficient mouse is indicated as band 4*. B, The intensities of two major in-frame bands (bands 4 and 5 in A) as well as those of two bands over-represented in donor CTLA-4-deficient T cells (bands 4* and 8) were calculated relative to the summed total of the intensities of all 10 bands on the indicated day following adoptive transfer.
profile (Fig. 6, first lane) shows a Gaussian intensity distribution, whereas there are a number of deviations in the pattern from the CTLA-4-deficient T cells (second lane).

The first example of a CTLA-4-deficient T cell expansion involves an out-of-frame TCR rearrangement identified as band 4 in the KO lane. It provides a nonselectable marker for a T cell line that has been positively selected on a functional TCR resulting from the rearrangement of the second chromosomal TCR β-chain locus. The advantage to analyzing bands representing out-of-frame, nonselected CDR3 lengths is that they are under-represented in the rearrangement assay and can be quantitated over a low background. The unusual intensity of this band is evident in the starting 50:50 mixture of adaptively transferred cells, but it falls to the level of the wild-type signal by day 25 posttransfer (Fig. 6B, top panel).

The second example of a CTLA-4-deficient T cell expansion is represented by an in-frame band on the short end of the CDR3 length distribution (band 8). Changes in band intensities on the edges of the distributions are easier to appreciate than those occurring in the central lengths. For comparison’s sake we show the relative intensity of the two bands flanking band 4*, bands 4 and 5, which appear to be part of the Gaussian distribution (Fig. 6B, lower panel). The relative intensities of bands 4 and 5 change little with time. The small increase on day 25 may reflect the loss of autoreactive CTLA-4-deficient T cells. These data suggest that wild-type cells caused the selective elimination of CTLA-4-deficient cells following adoptive transfer and thus prevented the development of autoimmune disease. This is consistent with a mechanism in which CTLA-4 functions nonintrinsically to induce peripheral tolerance via the deletion of activated self-reactive cells.

An interesting phenomenon is observed on day 2, when additional changes are observed in the CDR3 length intensity profile that are not representative of either input population. We interpret these as temporary shifts in the T cell population due either to direct interactions between the wild-type and CTLA-4-deficient splenocytes or to the reaction of the CTLA-4-deficient cells to the environment of the new host. These shifts can also be observed in recipients of CTLA-4-deficient cells alone, in which the skewed distribution of bands on day 25 differs from that of the original cells (data not shown).

Both approaches measuring the presence of CTLA-4-deficient T cells in the animals receiving the 50:50 mixture show a loss of the CTLA-4-deficient T cells relative to the wild-type cells. The presence of similar numbers of peripheral lymphocytes in recipients of either mixed or only wild-type cells leads to the conclusion that the CTLA-4-deficient cells were being eliminated.

**Discussion**

CTLA-4-deficient mice develop a fatal lymphoproliferative disease accompanied by lymphocytic infiltration and destruction of a number of different organs. Since the disease is Ag specific (11, 24–27), and thymic development is normal in the absence of CTLA-4 (10, 11), disease is thought to result from a defect in peripheral tolerance to self Ags. These peripheral Ags represent molecules that either were ignored during thymic negative selection or interacted with sufficiently low avidity that reactive T cells escaped central tolerance. In either case the interaction of such T cells with the self Ag in the periphery without concomitant inflammatory signals would lead to elimination of the T cells or perhaps convert a subpopulation into regulatory cells. There are a number of models in which the absence of CTLA-4 can be envisaged as leading to the autoimmune phenotype of the CTLA-4-deficient mice. In the first of these, the action of CTLA-4 is intrinsic to inactivation or elimination of such cells (Fig. 7A). This model is consistent with the function of CTLA-4 in strong, high

**FIGURE 7.** Possible mechanisms of action of CTLA-4. A, CTLA-4 acts directly upon self-reactive T cells. T cells stimulated by self Ag and CD28 become activated, but normally are inhibited by CTLA-4 expressed on the cell surface. In the absence of CTLA-4, self-reactive cells remain activated, infiltrate a variety of organs, and cause tissue destruction. Since CTLA-4 must be present on the autoreactive T cell itself to be inhibitory, the additional presence of CTLA-4-expressing T cells should not influence disease development. B, CTLA-4 is necessary for the development or maturation of regulatory cells, which normally keep self-reactive T cells in check. CTLA-4 engagement causes a population of cells to develop into regulatory cells. Alternatively, CTLA-4 engagement may cause some cells to induce others to become regulatory cells. Since the addition of wild-type Thy 1.2+ T cells alone ameliorates disease, the regulatory cells here are most likely regulatory T cells. The regulatory cells inhibit the function of autoreactive cells either by direct protein-protein interactions or indirectly, such as by the secretion of inhibitory cytokines. In the absence of CTLA-4, the regulatory cell population does not develop, and self-reactive T cells are not inhibited. C, Autoreactive T cells are down-regulated by binding to CTLA-4 on the surface of other cells. In the absence of CTLA-4, regulatory cells cannot bind self-reactive T cells, which receive no down-regulatory signal from their B7-like molecules. Alternatively, a regulatory cell may bind an autoreactive T cell via CTLA-4 and inhibit the T cell by means other than signaling through the B7-like molecule.
affinity, costimulation-dependent immune responses. Alternatively, the autoreactive cells could be affected indirectly by CTLA-4. Most likely, this would be through the actions of regulatory cells that require CTLA-4 for development (Fig. 7B) or for their actual function (Fig. 7C).

Our data as well as those reported by Bachmann et al. (20) do not support the first model of intrinsic down-regulation by CTLA-4 during the establishment of peripheral tolerance. However, Greenwald et al. (26) reported that CTLA-4-deficient TCR transgenic T cells that have been adaptively transferred into wild-type mice lose their ability to be tolerized by high dose peptide immunization. As the TCR transgenic, CTLA-4-deficient T cells were exposed to Ag in the presence of wild-type T cells, these results would appear incompatible with those reported here. We suggest that there are significant differences in the nature of Ag presentation in the two experimental systems. The first and perhaps greatest difference is the nature of the T cell stimulus. It is not clear that in situ recognition of tissue-specific self Ags, as opposed to the recognition of an i.v. delivered exogenous Ag with strong avidity for the transgenic TCR, is easily comparable. In the CTLA-4-deficient mice, many of the autoreactive T cells in the periphery would necessarily be of low affinity for diverse self Ags, since central tolerance would have caused the deletion of those cells with high affinity for self. Second, in our experiments many of the adaptively transferred T cells were already activated, whereas Greenwald et al. (26) activated the cells following adoptive transfer. Third, our functional assay for the induction of tolerance, the development of autoimmune disease, was performed in the presence of wild-type T cells, whereas the TCR transgenic, CTLA-4-deficient T cells described by Greenwald et al. were assayed in vitro in the absence of wild-type T cells. Finally, it is likely that the number of appropriate regulatory cells that were present differed in these two experimental systems. In our adoptive transfer model, the 50:50 mixture would include a sufficient number of endogenous self-Ag-specific regulatory T cells. It should be pointed out that a 25:75 mixture was already insufficient to guarantee survival. In the DO11 system regulatory cells might not be present in sufficient numbers to induce tolerance in a large number of high affinity T cell clones. In fact, it has been reported that TCR transgenic, RAG-deficient mice have low levels of regulatory T cells (30). The data reported by Greenwald et al. (26) show that CTLA-4 has intrinsic effects on the course of a T cell response. Our data indicate that in the CTLA-4-deficient mice these effects are taking place in a regulatory cell population and not the autoreactive cells directly responsible for the disease phenotype.

Bachmann et al. (20) demonstrated that irradiated mice reconstituted with chimeric bone marrow from wild-type and CTLA-4-deficient mice failed to develop the fatal disease of CTLA-4-deficient mice. These experiments showed that wild-type bone marrow-derived cells were capable of inhibiting either the development or function of autoreactive CTLA-4-deficient cells. Our data further indicated that wild-type Thy 1.2+ cells could inhibit the destructive function of activated, mature CTLA-4-deficient Thy 1.2+ cells. Recent experiments have also shown that Ag-specific, CTLA-4-deficient T cells in mice reconstituted with chimeric bone marrow undergo activation, expansion, and deletion in response to infection indistinguishably from wild-type T cells in the same animal (21). These data support our observation that wild-type T cells can control the function of activated CTLA-4-deficient T cells.

We propose that the absence of CTLA-4 leads to autoreactive T cell activation because CTLA-4 is required for the generation or function of a regulatory T cell compartment. It is not yet clear whether this would be one of the currently recognized compartments or an as yet unknown population. A similar regulatory mechanism has been proposed for the control of autoreactive T cells by IL-2. IL-2 is an important growth factor for T cells, yet mice deficient in IL-2 or the α- or β-chain of the IL-2R have an increased number of activated T cells and develop a spontaneous autoimmune-like disease. It has been shown that IL-2R signaling is necessary for the induction of regulatory T cells and the ultimate elimination of self-reactive T cells (31). IL-2 may function by inducing the expression of CTLA-4, which, in turn, is necessary for the development of active regulatory T cells. Alternatively, CTLA-4 may be required for the induction of regulatory cells via an IL-2-independent mechanism. This would imply that autoreactive T cells in the periphery could be inactivated or eliminated by two different pathways, perhaps using independent sets of regulatory cells. This model is also consistent with the results reported by Salomon et al. (32), who observed that spontaneous diabetes was exacerbated in B7-1/B7-2-deficient and CD28-deficient mice on the nonobese diabetic background. These mice exhibited a lack of CD4+CD25+ regulatory T cells, and the development of diabetes could be inhibited through the transfer of CD4+CD25+ regulatory T cells from control nonobese diabetic mice. In addition, Read et al. (33) and Takahashi et al. (34) have recently shown that CTLA-4 engagement is necessary for the function of mature CD25+ CD4+ regulatory T cells in controlling autoimmune intestinal inflammation.

It will be interesting to determine whether the regulatory cells are Ag specific. This could be tested using T cells from TCR transgenic, RAG-deficient mice as a source of regulatory cells. Cells from such mice have been shown to be deficient in regulatory activity (30).

Evidence that CD4+CD25+ regulatory cells also express CTLA-4 is suggestive of a model in which CTLA-4 directly binds to ligands on autoreactive T cells, thus causing their inhibition (Fig. 7C). Since T cells have been shown to express B7-1 and B7-2 (35, 36), binding to CTLA-4 could be mediated by CD80 or CD86 or by another B7-like molecule. The role of B7 molecules on T cells is not currently clear, although there is evidence that T cell CD80:CTLA-4 interactions can induce anergy in T cells exposed to Ag in the absence of sufficient costimulation (37). Binding of this cell surface molecule could cause the autoreactive T cell to be inhibited or deleted.

The data presented here do not allow us to distinguish between a requirement for CTLA-4 for the development or function of regulatory T cells, and the two are not necessarily mutually exclusive. We can conclude that CTLA-4 functions via an indirect mechanism to inhibit fatal tissue destruction by autoreactive T cells in the periphery. While it is possible to imagine more complex models of CTLA-4-mediated inhibition involving additional steps and cell types, the models presented above are straightforward and therefore more amenable to further testing.

In summary, we have shown that the role of CTLA-4 in the establishment of peripheral tolerance is more complex than the simple transmission of inhibitory signals into self-reactive T cells. A number of models can be envisaged to explain the data presented. Future experiments will more fully characterize how this more complex regulation is accomplished and whether it is mediated by specific regulatory T cells.

References
CTLA-4 ACTS INDIRECTLY IN ESTABLISHMENT OF PERIPHERAL TOLERANCE


