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Differential Role of CTLA-4 in Regulation of Resting Memory Versus Naive CD4 T Cell Activation

D. P. Metz,* D. L. Farber,† T. Taylor,* and K. Bottomly2*

Regulation of peripheral T cell responses is critical for preserving self tolerance. Memory T cells have a lower threshold for activation through the TCR and are thought to be less dependent on costimulation than naive T cells, suggesting a requirement for more stringent regulation of memory T cells. We have recently shown that CD4 engagement apart from the TCR results in the inactivation of memory, but not naive, CD4 T cells. We show here that this inhibition requires ligation of CTLA-4, in that blocking CTLA-4-B7 interactions restores memory CD4 T cell responsiveness. Early signaling through CTLA-4 is possible because resting memory, but not naive, CD4 T cells contain intracellular stores of CTLA-4 that are continuously recycled between the cytoplasm and the cell surface. This mechanism ensures that low intensity TCR engagements, which are thought to be important for peripheral T cell longevity, do not cause memory T cell activation but instead raise their threshold for costimulatory signals. This may give memory T cells an extended lifespan with a reduced risk of inappropriate activation. The Journal of Immunology, 1998, 161: 5855–5861.

Interactions of peripheral CD4 T cells with MHC class II-positive APCs in the absence of exogenous Ags are required for long term T cell survival (1, 2). To prevent proliferation to self peptides, activation of resting T cells has to be stringently regulated. For naive CD4 T cells, tolerance to self is assured by their need for two signals to proliferate, one via contact of Ag/MHC with the TCR and the second via contact of B7 molecules expressed on APCs with a costimulatory molecule, such as CD28 (3, 4). The need for costimulation ensures that naive T cells become activated only when APCs are loaded with exogenous Ag because resting APCs express few or no B7 molecules (5). In the absence of costimulation, naive CD4 T cells become unresponsive (6).

Memory CD4 T cells develop during a primary infection and, like naive T cells, are resting, long-lived lymphocytes. However, unlike naive CD4 T cells, memory T cells are previously primed cells and have been reported to be more sensitive to triggering through the TCR than naive T cells (7, 8), to respond more vigorously to Ag in terms of proliferation (9) and cytokine production (10), and to exhibit altered coupling of proximal signaling events to the TCR (11). Furthermore, while naive T cells recirculate between blood and secondary lymphoid organs, memory CD4 T cells are capable of entering nonlymphoid tissue directly from the blood (12, 13). The altered recirculation pattern enhances the chance of contacting foreign Ag, but also exposes memory T cells to new self peptides for which they may not have been selected in the thymus. While the altered signaling and recirculation pathways are believed to contribute to a more vigorous secondary immune response, they also pose the question of how memory T cell responses are regulated and how proliferation due to recognition of self peptide is prevented.

While it is established that naive T cell responses to self are controlled by their requirement for costimulation, the role of costimulation in regulating memory T cell responses is still unclear. It has been shown that unlike naive T cells, resting, Ag-primed CD4 T cells respond to Ag presented by resting B cells that do not express costimulatory molecules (14). Furthermore, in vitro stimulated memory CD4 T cells, but not naive T cells, proliferate to plate-bound anti-CD3 in the absence of additional costimulatory molecules (15). Several in vivo studies using CTLA4-Ig to block costimulation delivered through B7 also indicate that memory T cells are costimulation independent (16, 17), while other studies suggest that a secondary response can be blocked by CTLA4-Ig in vivo (18). It is possible that the discrepancies in these studies resulted from the inability to deliver CTLA4-Ig efficiently in the former studies or that the requirement of memory CD4 T cells for costimulation depends on the circumstances of activation.

CTLA-4, a CD28 homologue that negatively regulates the proliferation of effector T cells (19, 20), has recently been shown to prevent costimulation through CD28 when APCs express limiting B7 molecules. The importance of CTLA-4 in regulating effector T cell responses is emphasized by the massive lymphoproliferation observed in mice that lack CTLA-4 (21, 22). A role for CTLA-4 in the regulation of memory CD4 T cell responses in mice has recently been suggested (23) but has not yet been demonstrated.

We have recently shown that CD4 ligation in memory, but not naive, CD4 T cells leads to unresponsiveness (24, 25). In these studies memory CD4 T cells failed to proliferate to soluble anti-CD3 presented by syngeneic MHC class II+ APCs. The response was restored when anti-CD3 was presented by APCs that did not express MHC class II or expressed a mutant MHC class II molecule unable to engage CD4. We hypothesized that memory, but not naive, CD4 T cells fail to proliferate under conditions that do not recruit CD4 into the TCR complex, but instead allow independent CD4-MHC class II interactions, which resemble the inhibition produced in response to Ab-mediated CD4 cross-linking. Such a mechanism may be important in preventing memory CD4 T cells from proliferating to self peptides that fail to recruit CD4 to the TCR interaction site.

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To determine the role of costimulation in activation of resting memory CD4 T cells, we investigated the role of CD28/CTLA-4 in memory T cell responses. We show here that both naive and memory CD4 T cell proliferation in response to soluble anti-CD3 requires costimulation through CD28. We further show that under conditions where CD4-MHC class II interactions occur, memory CD4 T cells have a higher threshold for costimulation through CD28 than do naive T cells. Furthermore, the inability of memory CD4 T cells to proliferate in the presence of CD4-MHC class II interactions is dependent on CTLA-4-B7 interaction early after activation. CTLA-4 functions uniquely in memory CD4 T cells in that it continuously recycles from intracellular stores to the cell surface. We propose that the ability of CTLA-4 signaling to inhibit memory CD4 T cell responses is dependent on CD4 ligation. This mechanism ensures that low intensity TCR engagement, which mediates CD4-MHC class II engagement away from the TCR, does not cause memory T cell activation, but instead raises their threshold for costimulatory signals. This may extend the life span of memory CD4 T cells with reduced risk of inappropriate activation.

Materials and Methods

Mice

Female BALB/c ByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained at the animal facility at Yale University (New Haven, CT). All mice were used at 8–12 wk of age. MHC class II-negative mice were originally obtained from Dr. D. Mathis (Institut de Chimie Biologique, Strasbourg, France) (26) and were bred and maintained at the Yale animal facility.

Cell lines

DAP.3 fibroblasts and DAP.3 transfected with I-A\(^{d}\) (line 44) were gifts from Dr. R. Germain (27). The fibroblasts were cotransfected with FcRII by Dr. D. L. Farber as recently described (24).

Antibodies

The following mAbs were purified from supernatants of hybridomas maintained in this laboratory using standard protein A or protein G affinity chromatography: anti-CD3e (rat IgG2c, C363.29B), anti-CD4 (rat IgG2b, GK1.5), anti-CD8 (rat IgG2b, 2.43), anti-CD8 (rat IgG2a, 53-6.72), anti-Thy-1 (rat IgG2c, Y19), anti-I-A\(^{d}\) (mouse IgG2a, 212.A1), and anti-murine FcRII (rat IgG2b, 2.4G2). Purified as well as labeled mAbs to anti-CD45RB (rat IgG2a, C353.16A), CTLA-4 (Armenian hamster IgG, UC10-4F10), isotype control (pooled Armenian hamster IgG, G94-56), CD28 (anti-mouse IgM-coated magnetic beads (Collaborative Research, Bedford, MA). CD4 T cells were collected by magnetic depletion of Ab-labeled cells from the internalization of CTLA-4/PE3 mAb, parallel cultures were left out plate-bound anti-CD3 and anti-CD28 at 37°C in the presence of either phycoerythrin-labeled anti-CTLA-4 or an isotype control mAb for 3 h. To distinguish between CTLA-4 cell surface staining and staining resulting from the internalization of CTLA-4/PE \(^{3}\) mAb, parallel cultures were left unstained during the culture period and were cell surface labeled with anti-CTLA-4/PE or the isotype control for 30 min on ice after culture. Cells were analyzed by flow cytometry for the uptake of CTLA-4 mAbs.

Results

Proliferation of memory CD4 T cells to soluble anti-CD3 bound to FcRs on fibroblasts is costimulation dependent

Memory CD4 T cells proliferate to plate-bound anti-CD3, while naive T cells fail to do so, suggesting that memory T cells proliferate in the absence of costimulation (15). However, both naive and memory CD4 T cells respond equally well to soluble anti-CD3 when expressed on fibroblasts (DAP.3) transfected with Fc-\(\gamma\) receptor II (Fc-\(\gamma\)RII). In this instance naive CD4 T cells are capable

\(^{3}\) Abbreviations used in this paper: PE: phycoerythrin; Fc-\(\gamma\)RII; Fc-\(\gamma\) receptor II.
of proliferating because costimulation is provided by the fibroblasts in the form of B7.1. To determine whether memory CD4 T cells can proliferate in the absence of costimulation, proliferation assays were performed either in the presence of CTLA4-Ig or in the presence of fibroblasts that had been sorted for a lack of B7.1 expression. Interestingly, proliferation of naive and memory CD4 T cells was equally blocked when stimulated in the presence of B7.1+ fibroblasts and CTLA4-Ig (Fig. 1A) or in the presence of B7.1− fibroblasts (Fig. 1B). The importance of costimulation was further accentuated by the fact that both T cell populations proliferated when Abs to CD28 were added to cultures containing B7.1+ fibroblasts. These data clearly show that naive and memory CD4 T cells are equally dependent on costimulation when anti-CD3 is expressed on FcRs of APCs.

CD28 ligation can overcome memory CD4 T cell unresponsiveness to anti-CD3 in the presence of syngeneic APC/II−

Previous studies have shown that unlike naive CD4 T cells memory T cells fail to proliferate to anti-CD3 in the presence of syngeneic MHC class II+ APCs (APC/II+). Hyporesponsiveness was associated with a lack of cytokine production (24) and an inability to enter cell cycle (25). The incapacity of memory CD4 T cells to proliferate was dependent on an interaction of CD4-MHC class II, since responses were restored when APCs were derived from MHC class II-negative mice or from fibroblasts transfected with MHC class II that was mutated at the CD4 binding sites (29). It was proposed that memory T cells fail to become activated upon CD4-MHC class II interaction in situations where weak TCR stimulation occurs and CD4 is not efficiently recruited to the TCR complex (25). Since the costimulation requirements of memory CD4 T cells in the presence of CD4 ligation might differ, we investigated the potential role of CD28 in this response. Naive and memory CD4 T cells were stimulated with soluble anti-CD3 presented by APC/II− (Fig. 2) shows that both memory and naive CD4 T cells proliferated in response to soluble anti-CD3 expressed on APC/II+ in the presence or the absence of anti-CD28 mAb (Fig.

FIGURE 1. Memory and naive CD4 T cells fail to proliferate in response to soluble anti-CD3 presented by FcR-transfected fibroblasts in the absence of costimulation through CD28. A, Proliferation in the presence of B7.1+ fibroblasts with or without soluble CTLA4-Ig and anti-CD28. B, Proliferative response of memory and naive CD4 T cells to soluble anti-CD3 in the presence or the absence of anti-CD28 presented by fibroblasts lacking B7.1 expression. Assays were conducted in triplicate, and mean values are indicated ± SEM.

FIGURE 2. Unresponsiveness of memory CD4 T cells to soluble anti-CD3 expressed on syngeneic APC/II− can be reversed by Ab-mediated CD28 ligation. Memory and naive CD4 T cells were stimulated with soluble anti-CD3 presented by APC/II+ (A) or APC/II− (B) in the presence or the absence of anti-CD28. Results represent the mean ± SEM from triplicate wells. C, FACS analysis of B7.1 and B7.2 expression on resting T-depleted splenocytes isolated from MHC class II+ and MHC class II− mice.
While B7 expression on MHC class II increased the threshold for costimulation dependent on costimulation than is the proliferation of naive T cells. The proliferation of memory CD4 T cells in response to anti-CD3 is more dependent on the level of B7.1 expression (Fig. 2A). In contrast, naive, but not memory, CD4 T cells proliferated in response to anti-CD3 in the presence of resting APCs (Fig. 2A). mAb-mediated CD28 ligation restored proliferation of memory CD4 T cells in the presence of APCs and enhanced the proliferation of naive T cells (Fig. 2A). The difference in the ability of APCs and APCs to induce memory T cell responses was not due to differences in B7 expression, since APCs and APCs expressed similarly low levels of B7.1 and B7.2 (Fig. 2C). Although B7 expression was increased in both APC populations later in the response (data not shown), late expression of high B7 on the APC is unlikely to be involved in the initiation of the T cell response. We conclude that in the presence of resting APCs, but not APCs, the proliferation of memory CD4 T cells in response to anti-CD3 is more dependent on costimulation than is the proliferation of naive T cells.

MHC class II expression on APCs increases the threshold for costimulation in memory CD4 T cell responses to anti-CD3

While B7 expression on MHC class II and class II APC was the same, CD28-B7 interactions were only sufficient to activate memory CD4 T cells when APCs lacked MHC class II. Yet the same CD28-B7 interactions induced naive CD4 T cell responses when APCs expressed MHC class II. This suggested that memory CD4 T cells needed more costimulation than naive T cells when MHC class II was expressed on the APC. To test this hypothesis, we sorted MHC class II and class II fibroblasts for low and high expression of B7.1 and used these sublines to stimulate naive and memory CD4 T cells. Both populations proliferated in response to anti-CD3 in the presence of class II-negative fibroblasts independent of the level of B7.1 expression (Fig. 3B). However, in the presence of class II only naive CD4 T cells proliferated in response to anti-CD3 at low levels of B7.1. In contrast to the responses induced at low levels of B7.1, both naive and memory CD4 T cells responded in the presence of high B7.1 (Fig. 3A). Furthermore, the induction of unresponsiveness seen upon CD4/MHC class II interactions was abrogated; therefore, CD4 ligation due to MHC class II expression on APCs increases the threshold for costimulation on memory, but not naive, CD4 T cells. We concluded that memory CD4 T cells have a greater requirement for costimulation than naive T cells to overcome the inhibitory signal delivered through CD4-MHC class II interactions.

Memory, but not naive, CD4 T cells express intracellular stores of CTLA-4

It has recently been suggested that CTLA-4 signaling increases the threshold for costimulation through B7 molecules in activated T cells (20, 30). We sought to investigate the possibility that CTLA-4 signaling affected memory CD4 T cells at the initiation of a response to soluble anti-CD3. CTLA-4 has not been detected on resting T cells (31, 32). However, resting memory CD4 T cells represent previously activated cells that may retain residual expression of CTLA-4. We investigated this possibility by staining isolated resting naive and memory CD4 T cells for CTLA-4 expression. No cell surface expression of CTLA-4 was detected on naive or memory CD4 T cells (Fig. 4A). However, anti-CTLA-4 Western blots prepared from resting and 48-h activated naive and memory CD4 T cell lysates showed several bands between 33–37 kDa in the activated naive and memory T cell populations that were likely to represent differentially glycosylated forms of CTLA-4, as has been previously described (33). These bands were also visible in resting memory, but not resting naive, CD4 T cells (Fig. 4B), suggesting that resting memory CD4 T cells express intracellular stores of CTLA-4. Despite a Percoll gradient separation, we cannot rule out that some activated T cells contaminated the resting memory CD4 T cell pool used for Western blotting. To exclude this possibility we stained intracellularly for CTLA-4 and analyzed the data by flow cytometry, which allowed us to gate on low angle and sideways light scatter to exclude large activated T cells. Similar to the Western blot, resting memory CD4 T cells revealed a small amount of intracellular CTLA-4 (Fig. 4C). While the levels indicated by staining are low, no CTLA-4 is seen in naive CD4 T cells, and no signal is seen in memory T cells with the isotype control Ab. The prevalence of intracellular stores of CTLA-4 in memory T cells suggested that CTLA-4 may be available to resting memory CD4 T cells.
Resting memory CD4 T cells continuously recycle CTLA-4 between the cytoplasm and the cell surface. It has recently been shown that cell surface expression of CTLA-4 is unstable on activated T cells, and that CTLA-4 continuously recycles between endosomal compartments and the cell surface (32, 34). To determine whether CTLA-4 is recycled in resting memory T cells or whether TCR ligation induces CTLA-4 recycling, we incubated CD4 T cells in the presence of anti-CTLA-4/PE or an isotype control/PE mAb for 3 h at 37°C. Cells were stimulated with plate-bound anti-CD3 and anti-CD28 or remained unstimulated. If CTLA-4 cycles between the cell surface and endosomes, then labeled anti-CTLA-4 mAbs added to the cultures will accumulate intracellularly, as has recently been shown (31). To distinguish between reaccumulated CTLA-4 inside the cells and stable cell surface expression, duplicate cultures were labeled with anti-CTLA-4/PE or the isotype control mAb after culture for 3 h. Unlike resting naive T cells, a subpopulation of memory CD4 T cells was CTLA-4/PE positive after incubation with the mAb for 3 h, whereas no staining was observed with the isotype control mAb (Fig. 5A). Expression of CTLA-4 was enhanced when memory T cells were stimulated with plate-bound anti-CD3 and anti-CD28, while CTLA-4 expression was not observed in similarly activated naive CD4 T cells (Fig. 5B). Naive and memory CD4 T cells that were stained with anti-CTLA-4/PE after culture for 3 h did not stain for CTLA-4 (data not shown). The fact that memory CD4 T cells were positive for CTLA-4 when cultured in the presence of the mAb but not when anti-CTLA-4 was added after culture suggested that CTLA-4 continuously recycled between the cytoplasm and the cell surface, and that cell surface expression of CTLA-4 was unstable.

Blocking CTLA-4 interactions with B7 reinstates memory CD4 T cell responses to soluble anti-CD3 in the presence of B7low APC/II

To determine how CTLA-4 may function in the activation of memory CD4 T cells, we investigated memory CD4 T cell proliferation in response to soluble anti-CD3 in the presence of APC/IIo or APC/II1 under conditions where CTLA-4-B7 interactions were blocked by Fab of anti-CTLA-4. Blocking CTLA-4-B7 interactions had no effect on the proliferation of naive CD4 T cells in response to anti-CD3 in the presence of APC/IIo or APC/II1 (Fig. 6B). However, while memory CD4 T cell responses in the presence of APC/IIo were also unaffected by CTLA-4 Fab fragments (Fig. 6B), proliferation in the presence of APC/II1 was reinstated when CTLA-4-B7 interactions were blocked by anti-CTLA-4 Fab (Fig. 6A). These data suggest that at limiting amounts of B7, 1, CTLA-4 signaling prevents memory CD4 T cell proliferation in the presence of APC/II1.

FIGURE 5. In contrast to CD45RBhigh naive CD4 T cells, CD45RBlow memory T cells incorporate anti-CTLA-4 mAb, but not isotype control mAb, after culture for 3 h. Memory (A) and naive (B) CD4 T cells were stained with PE-labeled anti-CTLA-4 (solid line) or an isotype control mAb (dotted line) before culture at 37°C for 3 h. CD4 T cells either remained unstimulated (top panel) or were stimulated with plate-bound anti-CD3 and anti-CD28 (bottom panel). Staining was analyzed by flow cytometry. No staining was observed if T cells were stained after culture for 3 h (data not shown).
It has been shown that the strength of the TCR signal and the need for costimulation are inversely correlated; the weaker the TCR signal, the more CD28 ligation is required (23). Early signaling events occurring after T cell stimulation with soluble anti-CD3 presented by APCs are identical with those seen upon antagonistic peptide stimulation (35). In this instance, CD28 ligation is probably required to enhance the strength of the TCR signal. In contrast, plate-bound anti-CD3 and soluble anti-CD3 presented by FcR-transfected CHO cells (15), which induce memory CD4 T cell proliferation in the absence of costimulation, are likely to act as agonists, leading to extensive cross-linking of the TCR, which overrides the need for costimulation in memory, but not naive, CD4 T cells. Therefore, the strength of the TCR signal is probably involved in determining whether memory CD4 T cells proliferate in the absence of costimulation. This is in contrast to naive CD4 T cells, where a strong TCR signal may lower the amount of costimulation that is required but does not allow proliferation in the absence of costimulation.

The inhibitory signal delivered by CD4/MHC class II interaction to memory CD4 T cells (24) can be overcome by ligation of CD28 or by blocking CTLA-4-B7 interactions, suggesting that CTLA-4 signaling in memory CD4 T cells prevents costimulation through CD28 under conditions where B7 expression on the APC is limiting. Interestingly, CTLA-4 does not inhibit proliferation of memory CD4 T cells when CD4-MHC class II interactions are removed. This suggests that signaling through both CD4 and CTLA-4 is required to prevent memory CD4 T cell proliferation. Studies of CTLA-4 recycling in memory CD4 T cells in the presence or the absence of CD4 ligation do not suggest that CD4 influences CTLA-4 cell surface expression within the first few hours of activation (preliminary data, not shown); however, a small change in surface expression may have significant consequences. It is also possible that CTLA-4 activity is dependent on CD4 signaling, and that in the absence of CD4 ligation, CTLA-4 proceeds to recirculate but fails to become activated. Although little is known about CTLA-4 function, it has been shown that the cytoplasmic domain of CTLA-4 contains two SH2 binding domains that require phosphorylation for CTLA-4 to signal (19). It is feasible that CD4-associated p56

**FIGURE 6.** Memory CD4 T cell responses to soluble anti-CD3 in the presence of MHC class II can be rescued by inhibiting CTLA-4 signaling. Memory and naive CD4 T cells were stimulated with soluble anti-CD3 presented by B7.1low, MHC class II+ (A) or MHC class II+ (B) fibroblasts in the presence or the absence of anti-CTLA-4 (Fab). Data are expressed as the mean ± SEM from triplicate wells. Similar results were obtained when whole anti-CTLA-4 mAbs were used instead of Fab (data not shown).

but not APC/II”. We conclude that CD4/MHC class II interaction is essential for CTLA-4-induced unresponsiveness.

**Discussion**

Activation of peripheral T cells is stringently regulated to prevent proliferation to self. While mechanisms underlying the control of naive T cell activation have been elucidated using TCR-transgenic mice, little is still known about the regulation of memory T cell activation. In the present study we investigate mechanisms that regulate memory CD4 T cell responses. We have previously shown that CD4 ligation by MHC class II inhibits memory, but not naive, T cell proliferation in response to soluble anti-CD3, and we show here that this inhibition requires CTLA-4. We demonstrate that memory CD4 T cell responses to soluble anti-CD3, which has been shown to induce a weak TCR signal in the presence of APCs (23), require costimulation through CD28, suggesting that memory CD4 T cells, like naive T cells, require a second signal for activation in the presence of APCs. We further show that in the presence of CD4 ligation, memory, but not naive, CD4 T cell activation has an increased requirement for costimulation. This is supported by the fact that memory CD4 T cell responses can be rescued by Ab-mediated CD28 cross-linking or by high B7.1 expression on the APC. Furthermore, the unresponsiveness to anti-CD3 observed in memory CD4 T cells is mediated by CTLA-4, which continuously recycles in memory, but not resting, naive CD4 T cells. These data suggest that under conditions of weak TCR stimulation and low B7 expression, CD4 ligation occurs, leading to CTLA-4-induced unresponsiveness.

It has recently been shown that the lymphoproliferative disorders seen in CTLA-4-negative mice are mediated by CD4-positive, not CD8-positive, T cells (30). While the role of CD4 in CTLA-4 signaling was not defined, the study suggested that CTLA-4 plays a different role in CD4 vs CD8 T cell homeostasis. A role for CD4 in the maintenance of CD4 T cell homeostasis may lie in the fact that long term survival of mature peripheral
CD4 T cells is dependent on the expression of MHC class II molecules but not on the presence of specific Ag (1, 35, 39). This implies that peripheral T cells require TCR engagement with MHC class II loaded with self peptide. Given that memory T cells have a lower threshold for proliferation than naive T cells (40), there is a danger that memory CD4 T cells might become activated by the signals for their survival. It is likely that weakly cross-reactive self peptide, similar to antigenic peptide or soluble anti-CD3, does not recruit CD4 into the TCR-MHC class II interaction site. CD4 engagement away from the TCR complex may therefore induce CTLA-4-mediated unresponsiveness to prevent memory CD4 T cell proliferation to self peptide but allow memory T cell survival.

Furthermore, this model may have implications for HIV-infected memory T cells, where CD4 is similarly ligated outside the TCR complex by gp120. It has been shown that hyporesponsive gp120-positive CD4 T cells can be induced to proliferate when CD28 is ligated by mAb (41, 42). It would be of interest to see whether such T cells express CTLA-4.

Even though it is currently not clear how CD4 and CTLA-4 inhibit memory CD4 T cell responses, it is evident that the two mechanisms act in concert. We propose that CTLA-4 signaling is not just required for down-regulation of already activated T cells, but that it is a critical regulator of resting memory CD4 T cells. CTLA-4 inhibits memory CD4 T cell proliferation under conditions where CD4 is ligated outside the TCR complex.

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