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The Role of CTLA-4 in Regulating Th2 Differentiation

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To examine the role of CTLA-4 in Th cell differentiation, we used two newly generated CTLA-4-deficient (CTLA-4−/−) mouse strains: DO11.10 CTLA-4−/− mice carrying a class II restricted transgenic TCR specific for OVA, and mice lacking CTLA-4, B7.1 and B7.2 (CTLA-4−/−B7.1/B7.2−/−). When purified naive CD4+ DO11.10 T cells from CTLA-4−/− and wild-type mice were primed and restimulated in vitro with peptide Ag, CTLA-4−/− DO11.10 T cells developed into Th2 cells, whereas wild-type DO11.10 T cells developed into Th1 cells. Similarly, when CTLA-4−/− CD4+ T cells from mice lacking CTLA-4, B7.1, and B7.2 were stimulated in vitro with anti-CD3 Ab and wild-type APC, these CTLA-4−/− CD4+ T cells produced IL-4 even during the primary stimulation, whereas CD4+ cells from B7.1/B7.2−/− mice did not produce IL-4. Upon secondary stimulation, CD4+ T cells from CTLA-4−/− B7.1/B7.2−/− mice secreted high levels of IL-4, whereas CD4+ T cells from B7.1/B7.2−/− mice produced IFN-γ. In contrast to the effects on CD4+ Th differentiation, the absence of CTLA-4 resulted in only a modest effect on T cell proliferation, and increased proliferation of CTLA-4−/− CD4+ T cells was seen only during secondary stimulation in vitro. Administration of a stimulatory anti-CD28 Ab in vivo induced IL-4 production in CTLA-4−/− B7.1/B7.2−/− but not wild-type mice. These studies demonstrate that CTLA-4 is a critical and potent inhibitor of Th2 differentiation. Thus, the B7-CD28/CTLA-4 pathway plays a critical role in regulating Th2 differentiation in two ways: CD28 promotes Th2 differentiation while CTLA-4 limits Th2 differentiation. *The Journal of Immunology, 1999, 163: 2634–2639.

The proliferation and differentiation of T lymphocytes requires Ag, costimulation, and cytokines. The best-defined costimulators for T cells are B7.1 (CD80) and B7.2 (CD86), both of which are expressed on professional APCs, and the expression of which is increased by microbial pathogens and by various cytokines produced during innate immune reactions (1). B7 molecules bind CD28 on T cells and optimize T cell activation (2). A second receptor for B7 molecules, CTLA-4 (CD152), down-regulates T cell activation (3). CTLA-4 expression is up-regulated on activated T cells, and CTLA-4 binds B7.1 and B7.2 with higher affinity than does CD28 (4). The critical role of CTLA-4 in controlling T cell activation is dramatically illustrated by the fatal lymphoproliferative disease seen in CTLA-4-deficient (CTLA-4−/−) mice (5, 6). This disease requires CD4+ cells, suggesting an inhibitory role for CTLA-4 in the initiation and/or maintenance of CD4+ T cell responses (7).

The goal of the studies in this paper was to examine the role of CTLA-4 in T cell proliferation and differentiation. Much of our current knowledge of the function of CTLA-4 is based on experiments using agonistic and blocking Abs. Such studies have shown that CTLA-4 cross-linking inhibits cytokine secretion and cell cycle progression in T cells (8, 9). Many of these effects are opposite of the demonstrated effects of CD28, which promotes early cytokine (e.g., IL-2) production, T cell survival by inducing expression of anti-apoptotic genes (e.g., Bcl-xL), and T cell differentiation into effector cells (10). CD28 plays an important role in differentiation of naive CD4+ T cells and influences Th2 differentiation more than Th1 development (11, 12). The role of CTLA-4 in Th cell differentiation is not yet clear.

Although the phenotype of the CTLA-4−/− mouse strain has been informative, the early activation of CTLA-4−/− T cells and death of these mice by 4 wk of age have limited the analyses that can be done using this strain. To overcome these problems, we have used two approaches to examine the obligatory roles of CTLA-4 in CD4+ T cell activation and differentiation. We have bred the DO11.10 class II-restricted TCR transgenic mice with CTLA-4−/− mice, and compared Ag-specific responses of wild-type and CTLA-4−/− CD4+ DO11.10 T cells. To exclude in vivo activation of CTLA-4−/− T cells by as yet unknown self or foreign Ags, we also have bred the CTLA-4−/− mice with mice lacking B7.1 and B7.2 (13, 14) and analyzed the responses of these CTLA-4−/− CD4+ T cells to anti-CD3 Ab plus wild-type APCs. Both approaches show that if CD4+ T cells are activated by TCR engagement and costimulation in the absence of CTLA-4, the cells exhibit a dramatic tendency to differentiate into Th2 populations. Surprisingly, the absence of CTLA-4 has only a modest effect on T cell proliferation, and only during secondary responses in vitro. These results suggest there is reciprocal regulation of CD4+ Th cell differentiation by CD28 and CTLA-4. CD28 promotes Th2 differentiation, whereas CTLA-4 is a critical, potent inhibitor of this differentiation pathway.

Materials and Methods

Mice

Mice expressing the DO11.10 TCR transgene, which responds to the peptide residues 323–339 of chicken OVA in the context of I-Ak (BALB/c), were obtained from Dr. Dennis Loh (Roche, Nutley, NJ) (15). CTLA-4 heterozygous (+/−) mice were backcrossed onto the BALB/c background.
for six generations before they were bred with the DO11.10 TCR transgenic mice and intercrossed. PCR typing for the DO11.10 transgene was conducted using primers of sequence 5′-CAG GAG GGA TCC AGT GCC AGC-3′ and 5′-TG CTC TAC AGT GAG TGT G-3′. Genotyping of CTLA-4 was done by Southern blot analysis or PCR analysis of tail DNA for the wild-type locus and the neomycin resistance gene in the targeted locus (5). In addition, a combination of primers was used to amplify neo-

centric sequences from cultures of 10^5 cells/ml are shown. Symbols are as in Fig. 1B. Comparable proliferation. Results are representative of six experiments.

FIGURE 1. CTLA-4−/− DO11.10 T cells show wild-type levels of proliferation and increased IL-4 secretion during primary stimulation. A, A total of 4 × 10^6 CD4+/KJ1–26+/L-selectinhigh T cells were stimulated for 3 days with 2 × 10^5 APC and peptide concentrations as indicated. Prolif-

eration was measured by [3H]thymidine incorporation. Wild-type cells (plotted on the left, ▲) and CTLA-4−/− cells (plotted on the right, □) show comparable proliferation. Results are representative of six experiments. B, Cytokines were measured by ELISA in culture supernatants at 18, 30, 54, and 74 h after stimulation with 10 µg/ml peptide. IL-2, IFN-γ, and IL-4 concentrations from cultures of 10^5 cells/ml are shown. Symbols are as in A. Cells cultured without Ag did not produce measurable cytokines (data not shown).

In vivo administration of CD28

CTLA-4−/− B7.1/B7.2−/− or wild-type mice were injected i.v. with 100 µg of purified anti-CD28 Ab (PV-1) on days 0, 7, and 14 and sacrificed on day 19. Lymph node cells (2 × 10^7) were stimulated with various concentrations of anti-CD1 Ab (145-2C11), and cell cultures supernatants were harvested to measure cytokines at the indicated times.

Results

Proliferation and differentiation of CTLA-4−/− DO11.10 T cells

Wild-type DO11.10 and CTLA-4−/− DO11.10 mice show comparable thymic maturation of T cells and lymphocyte numbers early in life (data not shown). At 6–8 wk of age, CTLA-4−/− DO11.10 mice show mild lymphadenopathy and splenomegaly. This may be due to activation of T cells expressing TCRs other than the DO11.10 TCR, or activation of DO11.10 T cells by cross-reactive ligands. In contrast to nontransgenic CTLA-4−/− mice, which die by 3–4 wk of age, the CTLA-4−/− mice survive for >16 wk, presumably because the transgenic TCR reduces the frequency of autoreactive T cells.

To compare the responses of DO11.10 wild-type and CTLA-4−/− T cells, we purified CD4+/KJ1–26+/L-selectinhigh T cells from both strains. These T cells were activated in vitro with OVA23–33 and APCs, and proliferation and cytokine production were measured during primary and secondary responses. DO11.10 wild-type and CTLA-4−/− T cells showed comparable proliferation (Fig. 1A) and, in most experiments, similar IL-2 production.
whereas CTLA-4

Analysis of secondary responses showed that this is indeed the Ag, but are normally prevented from doing so by CTLA-4. produced detectable amounts of IL-4. This result suggests that naive T cells, restimulated as in Fig. 1A, show increased proliferation compared with wild-type cells (left, ▲), especially at high Ag concentrations. The same enhancement in proliferation was observed at days 1 and 3 after restimulation. Results are representative of six experiments. C, Cytokine secretion during restimulation was assayed as in Fig. 1B. CTLA-4−/− cells secrete high levels of IL-4, IL-5, and IL-10, whereas CTLA-4+/+ produce IFN-γ and IL-2.

FIGURE 2. CTLA-4−/− DO11.10 T cells show enhanced proliferation and a Th2 phenotype compared with wild-type DO11.10 T cells during secondary responses. A, DO11.10 T cells were activated for 4 days by culture with OVA peptide (10 μg/ml) and APCs, and viable cells were restimulated as in Fig. 1A. Proliferation was assayed on day 2, and cytokine levels were measured at the indicated times. CTLA-4−/− cells (right, □) show increased proliferation compared with wild-type cells (left, ▲), especially at high Ag concentrations. The same enhancement in proliferation was observed at days 1 and 3 after restimulation. Results are representative of six experiments. B, Cytokine secretion during restimulation was assayed as in Fig. 1B. CTLA-4−/− cells secrete high levels of IL-4, IL-5, and IL-10, whereas CTLA-4+/+ produce IFN-γ and IL-2.

during primary stimulation, and essentially no IFN-γ was produced, as expected from naïve T cells (Fig. 1B). The only consistent difference between these cell populations was that after 3–4 days of priming in vitro, the DO11.10 CTLA-4−/− T cells produced detectable amounts of IL-4. This result suggests that naive T cells are capable of producing IL-4 during primary responses to Ag, but are normally prevented from doing so by CTLA-4. If the absence of CTLA-4 allows IL-4 production during priming, then this should lead to Th2 responses upon restimulation. Analysis of secondary responses showed that this is indeed the case. Upon priming and restimulation with Ag and APCs, wild-type DO11.10 cells produced the Th1 cytokine IFN-γ, whereas CTLA-4−/− DO11.10 T cells produced Th2 cytokines, IL-4, IL-5, and IL-10 (Fig. 2B). This bias toward Th2 development in cells that lack CTLA-4 was observed over a range of peptide concentrations (0.1, 1, 10 μg/ml) during primary stimulation (data not shown). Interestingly, in these recall responses the CTLA-4−/− DO11.10 T cells proliferated more than wild-type DO11.10 cells (Fig. 2A), suggesting that the role of CTLA-4 in limiting T cell expansion is seen late during immune responses.

Cytokine production by T cells from CTLA-4−/− B7.1/B7.2−/− mice.

Although the experiments with DO11.10 T cells suggest that CTLA-4 regulates Th2 differentiation, an alternative possibility is that some of these cells are activated in vivo by either environmental or endogenous Ag, and this may affect subsequent differentiation of the T cells. It is difficult to exclude such in vivo activation by unknown Ags. We have shown that stimulation of CD28 is critical for T cell activation in CTLA-4−/− mice, and that T7 antagonists prevent “spontaneous” lymphocyte activation and disease in CTLA-4−/− mice (17). More recently, we have demonstrated that breeding the CTLA-4−/− mice with mice lacking both B7.1 and B7.2 also completely prevents endogenous activation and disease (13). T cells from CTLA-4−/− B7.1/B7.2−/− mice have the phenotypic and functional characteristics of naïve cells. We reasoned that studying the responses of CTLA-4−/− B7.1/B7.2−/− T cells to TCR cross-linking and wild-type APCs in vitro would provide an additional means of evaluating the functional capabilities of CTLA-4−/− T cells, without the confounding influence of in vivo activation.

CD4+ T cells were isolated from CTLA-4−/− B7.1/B7.2−/− mice and activated in vitro with anti-CD3 Ab and syngeneic wild-type APCs. The primary and secondary responses of these cells were compared with those of CD4+ T cells from mice lacking only B7.1 and B7.2. As was seen with DO11.10 T cells, the initial proliferation of these CTLA-4−/− B7.1/B7.2−/− T cells was similar to that of CTLA-4+/+ B7.1/B7.2−/− cells. However, these CTLA-4−/− cells proliferated more than the CTLA-4+/+ cells during secondary stimulation (data not shown). Strikingly, the CTLA-4−/− B7.1/B7.2−/− T cells produced IL-4, even during primary stimulation in the presence of wild-type APCs (Fig. 3A), whereas the CTLA-4−/+ B7.1/B7.2−/− cells did not. Moreover, upon secondary stimulation, these CTLA-4−/− cells secreted much more IL-4 and much less IFN-γ than did CTLA-4+/+ B7.1/B7.2−/− cells (Fig. 3B). This differentiation into Th2 cells did not appear to be solely determined by the strength of signal during the priming, because IL-4 was produced by CTLA-4−/− cells that had been primed over a range of anti-CD3 Ab concentrations (from 1:500 to 1:10,000) during priming (data not shown). Thus, when naïve T cells lacking CTLA-4 are exposed to an Ag analogue (anti-CD3 Ab) and costimulation, they differentiate into Th2 populations.

Neutralizing IL-4 during priming only partially inhibits Th2 differentiation of CTLA-4−/− T cells.

To investigate whether the Th2 differentiation of CTLA-4−/− T cells was due to IL-4 produced by CTLA-4−/− T cells or an intrinsic property of CTLA-4−/− T cells, we examined the consequences of priming CTLA-4−/− T cells in the presence of neutralizing anti-IL-4 Ab. CD4+/KJ1-26+/L-selectinhigh wild-type DO11.10 and CTLA-4−/− DO11.10 T cells were primed with peptide Ag and APC in the presence of a saturating concentration of anti-IL-4 Ab. During priming of CTLA-4−/− DO11.10 T cells with 10 μg/ml peptide for 5 days, the concentration of IL-4 in the supernatants was 0.07 ± 0.03 ng/ml, whereas in the presence of anti-IL-4 Ab no IL-4 was detectable (detection limit for IL-4 ELISA was <0.005 ng/ml). Therefore, IL-4 was effectively neutralized by the Ab. In both wild-type and CTLA-4−/− DO11.10 T cells, the presence of anti-IL-4 Ab during priming increased IFN-γ production upon secondary stimulation (Fig. 4). However, the presence of anti-IL-4 Ab during priming resulted in only a modest
decrease in IL-4 upon restimulation of CTLA-4−/− cells, compared with CTLA-4−/− cells primed in the absence of anti-IL-4. In secondary cultures, IL-4 was detected by ELISA in supernatants of CTLA-4−/− DO11.10 T cells that had been primed in the presence of anti-IL-4 (Fig. 4A). Furthermore, intracellular cytokine staining showed IL-4 production by CTLA-4−/− DO11.10 cells that had been primed in the presence of anti-IL-4 (Fig. 4B). In addition, IL-4 mRNA was detected by RNase protection assays (RPAs) of CTLA-4−/− DO11.10 cells that had been primed in the presence of anti-IL-4 (data not shown).

Similarly, the addition of anti-IL-4 Ab to the primary cultures of CTLA-4−/− B7.1/B7.2−/− CD4+ T cells failed to block IL-4 production during secondary stimulation of these CTLA-4−/− T cells(data not shown). Analyses of cytokine transcripts by RPAs gave similar results. When RPAs were performed with CTLA-4−/− B7.1/B7.2−/− and B7.1/B7.2−/− T cells that had been primed with anti-CD3 and wild-type APC in the presence of anti-IL-4 Ab and subsequently restimulated with plate-bound anti-CD3 Ab for 6 h, significant amounts of mRNA for the Th2 cytokines, IL-4, IL-5, IL-10, and IL-13 were only observed in the CTLA-4−/− B7.1/B7.2−/− T cells (data not shown). These data suggest that the propensity of CTLA-4−/− T cells to differentiate toward the Th2 pathway might be an intrinsic property of these T cells.

Anti-CD28 Ab stimulates Th2 differentiation in vivo in the absence of CTLA-4

The experiments described so far have investigated in vitro responses of CTLA-4−/− T cells to peptide Ag or TCR cross-linking and costimulation. We postulated that if CTLA-4 has an essential role in regulating Th2 differentiation, then providing mice lacking CTLA-4 with a CD28-mediated signal in vivo also might induce a Th2 response. To test this, a stimulatory anti-CD28 Ab was administered i.v. to CTLA-4−/− B7.1/B7.2−/− mice or wild-type mice, lymph node cells were harvested 19 days later, and IL-4 production was assayed following restimulation of lymph node cells with anti-CD3 Ab. We had previously shown that this anti-CD28 Ab (PV-1) produces a lymphoproliferative phenotype in CTLA-4−/− B7.1/B7.2−/− mice, but has no in vivo proliferative effect in wild-type mice (13). As shown in Fig. 5, exposure to this agonistic anti-CD28 Ab in vivo induced IL-4 production in CTLA-4−/− B7.1/B7.2−/− cells, but not in wild-type cells. The CTLA-4−/− cells made less IFN-γ than wild-type mice (data not shown). The lack of an effect of anti-CD28 Ab in wild-type mice suggests that the B7-CTLA-4 interaction can provide signals that limit CD28-mediated Th2 differentiation, even when CD28 is triggered with a cross-linking Ab.
The absence of CTLA-4 had no influence on initial proliferative responses and a relatively modest effect on proliferation upon restimulation. The modest effects of CTLA-4 on T cell proliferation are surprising in light of studies with Abs showing that CTLA-4 ligation limits T cell expansion very early after triggering (8, 9). In fact, our

FIGURE 5. In vivo anti-CD28 treatment of CTLA-4−/− B7.1/B7.2−/− mice induces IL-4 production. CTLA-4−/− B7.1/B7.2−/− and wild-type mice received 100 μg of either anti-CD28 Ab (PV-1) or control Ab on days 0, 7, and 14 and were sacrificed on day 19. A total of 2 × 10^6 lymph node cells were stimulated in vitro with anti-CD3, and culture supernatants were assayed for IL-4 production by ELISA after 2 days. Control hamster IgG has no effect in either mouse strain (data not shown). These results are representative of three experiments.
results suggest that CTLA-4 has an obligatory role in inhibiting T cell expansion late in immune responses. The kinetics of this effect are consistent with the kinetics of CTLA-4 expression, which typically increases 24–48 h after T cell activation in vitro (22).

We conclude from these studies that one of the major functions of CTLA-4 is to limit the magnitude of Th2 differentiation. When T cells recognize Ag on APCs, engagement of CD28 by B7 molecules promotes Th2 differentiation, whereas B7 binding to CTLA-4 blocks Th2 differentiation. The mechanism of these opposing effects of CD28 and CTLA-4 on cytokine profiles remains to be established. One potential mechanism could be that CTLA-4 competes with CD28 for B7 binding and therefore counteracts the Th2 differentiation induced by CD28 triggering. An alternative possibility is that CD28 transduces signals that directly activate the transcription of Th2 cytokine genes, and CTLA-4 inhibits these signals. We are currently comparing the effects of CD28 and CTLA-4 ligation on transcription factors that are known to bind to cytokine gene promoters (23) to identify the critical signals involved. Finally, these results raise the possibility that the skewed Th2 response of CTLA-4−/− T cells plays a role in the lymphoproliferative and infiltrative disease of CTLA-4−/− mice. Studies to define the roles of various cytokines in the disease of CTLA-4−/− mice, using cytokine antagonists and knockouts, are in progress.

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