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*J Immunol* 2002; 169:1182-1188; doi: 10.4049/jimmunol.169.3.1182
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CD40 Ligand and CTLA-4 Are Reciprocally Regulated in the Th1 Cell Proliferative Response Sustained by CD8\(^+\) Dendritic Cells\(^1\)

Francesca Fallarino, Ursula Grohmann, Carmine Vacca, Roberta Bianchi, Maria C. Fioretti, and Paolo Puccetti\(^2\)

Subsets of murine dendritic cells (DCs) from the spleen differ in their ability to induce proliferative responses in both primary and secondary CD4\(^+\) T cells. Recent evidence indicates that lymphoid-related CD8\(^+\) DCs fail to provide appropriate signals to freshly isolated secondary CD4\(^+\) T cells to sustain their proliferation in vitro. In the present study, we examined peptide-pulsed CD8\(^-\) and CD8\(^+\) DCs for ability to stimulate Th1 and Th2 cell clones with the same Ag specificity. Defective ability to induce proliferation was selectively shown by CD8\(^+\) DCs presenting Ag to the Th1 clone. The deficiency in CD8\(^+\) DCs was overcome by CD40 triggering before peptide pulsing. When exposed to CD8\(^+\) DCs in the absence of CD40 activation, the Th1 clone expressed low levels of CD40 ligand and high levels of surface CTLA-4. Neutralization of CTLA-4 during the DC/T cell coculture resulted in increased CD40 ligand expression and proliferation of T cells. Remarkably, the activation of CD40 on DCs under conditions that would increase Th1 cell proliferation, also resulted in down-regulation of surface CTLA-4. These results confirm differential effects of CD8\(^+\) and CD8\(^-\) DCs in the stimulation of Ag-presented Th cells. In addition, they suggest that reciprocal regulation of CD40 ligand and CTLA-4 expression occurs in Th1 cells exposed to CD8\(^+\) DCs. The Journal of Immunology, 2002, 169: 1182–1188.

\(^{1}\) Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand.

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DC preparation and treatments

DCs were prepared and fractionated according to CD11c/CD8α expression using positive selection columns in combination with CD11c and CD8α MicroBeads (Miltenyi Biotec, Bergish Gladbach, Germany), as described previously (13). Briefly, DCs were obtained from collagenase-treated spleens (collagenase type IV; Sigma-Aldrich, St. Louis, MO). Total spleen cells were then treated with EDTA to disrupt DC–T cell complexes according to a previously described procedure (27), and EDTA was also present in subsequent steps involving the use of positive selection columns. Cells were resuspended in a 1.080 g/cm³ isosmotic Nycodenz medium (Sigma-Aldrich), and centrifuged at 3000 rpm for 15 min at 4°C. The low-density fraction at the interface was collected and washed several times. The recovered cells were incubated with CD11c MicroBeads and separated using a positive selection column. Cells were then resuspended in RPMI 1640 medium supplemented with 10% FCS and allowed to adhere for 2 h, and this was followed by an additional 18-h incubation to allow DCs to detach. The recovered cells were routinely 96–98% CD11c+ and appeared to consist of 90–95% CD8+ and 5–10% CD8- cells. For preparation of CD8+ and CD8- fractions, the purified DCs were separated using positive selection columns and CD8α MicroBeads. After cell fractionation, the recovered CD8+ cells were ~45% CD4+ and typically contained ~0.5% contaminating CD8+ DCs, whereas the CD8- fraction was made up of >95% CD8+ DCs. The CD8+ DC fraction consisted of >95% B7-1+, >98% B7-2+, and >98% CD40+ cells, whereas the CD8- fraction consisted of >98% B7-1+, >99% B7-2+, and >98% CD40- cells. In all CD40 stimulations (11–13), DCs were incubated on ice for 10 min in PBS plus 10% mouse serum and washed 20 min with hamster anti-mouse CD40Ab (5 μg/ml), and then overnight at 37°C with goat anti-hamster Ab (5 μg/ml) in Iscove’s medium plus 10% FCS. CD40 ligation on DCs routinely involved the use of the second cross-linking Ab, as the latter appears to be necessary for effective DC activation. To check for possible nonspecific effects of anti-CD40 ligation, appropriate controls included incubation of the CD8+ DCs in the presence of the second Ab alone, which treatment appeared to be devoid of any functional effect. As an additional control, isotype-matched anti-mouse H-2Kd 31-3-4S, capable of binding to DCs, was also used in place of the primary anti-CD40 reagent (11).

Th cell clones

T cell clones F76 (Th1) and F2 (Th2) were derived by limiting dilution of cultured cells from the popliteal lymph nodes of DBA/2 mice immunized with P815AB-pulsed DCs as described elsewhere (28) and were maintained by weekly restimulation of 1 × 106 cells with 5 μM P815AB peptide and 6 × 104 irradiated spleen cells in complete medium containing 40 U/ml human rIL-2.

T cell proliferation assay

Assays were performed in triplicate in flat-bottom 96-well microtiter plates in a total volume of 200 μl. Cultures contained T cell clones (5 × 103 cells/well), purified DCs (at the indicated concentrations), and 5 μM P815AB peptide at concentrations of 20 μM with hamster anti-mouse CD40Ab (5 μg/ml), and then overnight at 37°C with goat anti-hamster Ab (5 μg/ml) in Iscove’s medium plus 10% FCS. CD40 ligation on DCs routinely involved the use of the second cross-linking Ab, as the latter appears to be necessary for effective DC activation. To check for possible nonspecific effects of anti-CD40 ligation, appropriate controls included incubation of the CD8+ DCs in the presence of the second Ab alone, which treatment appeared to be devoid of any functional effect. As an additional control, isotype-matched anti-mouse H-2Kd 31-3-4S, capable of binding to DCs, was also used in place of the primary anti-CD40 reagent (11).

Cytokine determinations in T cell cultures

Cultures were established using 5 × 104 T cells and 5 × 103 DCs in a 0.2-ml volume in the presence of 5 μM P815AB peptide, and supernatants were harvested at 24 h (for IL-2) or 48 h (for IFN-γ and IFN-α) for evaluation of cytokine contents by sandwich ELISA, as previously reported (8, 28). Briefly, IL-2 was measured by the use of mAb JES6-1A12 and biotinylated 145-2C11 mAb. IFN-γ levels were measured using R4-6A2 and biotinylated 4F10 mAb. IL-4 levels were measured using mAb G4-202 and biotinylated MR1 mAb and PE-conjugated hamster IgG. Samples were analyzed on a FACScan (BD Biosciences, San Jose, CA) and data were analyzed using Lysis II software (BD Biosciences). Live cells were selected for analysis using forward vs. side scatter gating.

Results

Differential ability of DC subsets to stimulate proliferation of Th1 and Th2 clones

Previous studies have documented differential effects of CD8+ and CD8- DCs in the proliferative response of freshly harvested secondary T cells with specificity for a synthetic influenza virus-related peptide or infectious virus (18). We wanted to assess any possible differential effects of DC subsets on the proliferation of Th1 and Th2 cell clones with specificity for a synthetic tumor/self-peptide, P815AB (28). Cultures were established using Th1 (F76) and Th2 (F2) cells in combination with fractionated CD8+ and CD8- splenic DCs in the presence of cognate peptide. Proliferation of T cells was determined at different times (i.e., 24, 48, 72, and 96 h), showing that maximum proliferation would occur at ~48 h (data not shown). As depicted in Fig. 1A, which reports the 48-h data using a range of DC concentrations, the CD8+ and CD8- DCs showed comparable ability to support the growth of Th2 cells. In contrast, significant proliferation of the Th1 clone was only observed when these cells were incubated with CD8- DCs. When cytokine levels were measured in culture supernatants (28), the Th2 cells appeared to express high levels of IL-4 and IL-2 regardless of the DC subtype added to the coculture as APCs. In contrast, the Th1 cells released significant amounts of IL-2 in addition to IFN-γ only in the presence of CD8- DCs (Fig. 1B). It should be noted that the Th1 clone did produce considerable levels of IFN-γ even when cocultured with CD8+ DCs. This finding emphasizes the inability of Th1 cells to proliferate in the presence of CD8+ DCs even when producing IFN-γ.

CD40 activation on CD8+ DCs enables these cells to support the growth of the Th1 clone

Using an in vivo model of P815AB presentation for induction of CD4+ T cell-dependent skin test reactivity, we have previously shown that the failure of peptide-loaded CD8+ DCs to initiate T cell reactivity in vivo may be overcome by activation of CD40 in vitro before transfer into recipient hosts (12). We therefore became interested in ascertaining whether CD40 ligation would overcome the inability of CD8+ DCs to support the growth of the Th1 clone

biological activity by measuring its ability to confer resistance to vesicular stomatitis virus infection upon L929 cells in the presence of neutralizing anti-mouse IFN-γ mAb R-6-6A2 (29). The activity of the supernatants was determined by comparison to that of rIFN-γ (ICN Pharmaceuticals, Basingstoke, U.K.). Results are expressed as international IFN units (IU).

The production of IFN-γ by DCs was assessed by ELISA as indicated above using overnight cultures of fractionated DCs (106) incubated in the presence or absence of anti-CD40 mAb. Aliquots of the same culture supernatants were also assayed for IL-12 p70 contents by ELISA as described elsewhere (11).

Cytokine production by DCs

DC culture supernatants were tested for IFN antiviral activity using a cytopathic effect reduction bioassay as previously described (29). DCs (1 × 106) were plated in each well of a 24-well culture plate in a volume of 1 ml in the presence or the absence of anti-CD40 mAb. Cultures were incubated at 37°C in 5% CO2 for 18 h, after which the supernatant was harvested. Aliquots of each sample (100 μl) were assayed for IFN-αβ
in vitro in the presence of cognate peptide. Cultures were established as illustrated above using Th1 or Th2 cells incubated with either type of peptide-loaded DCs, which were used either as such or after activation of CD40 in vitro, as previously described (11–13). Fig. 2A shows that CD40 activation had no effect on the proliferative response of Th2 cells incubated with CD8+ or CD8− DCs. Similarly unchanged was the proliferative response of Th1 cells exposed to CD8− DCs. In contrast, CD40 activation greatly increased the ability of CD8+ DCs to support the growth of the Th1 clone. Remarkably, the proliferation of the latter cells, namely, F76 clone cells exposed to CD8− activated CD8+ DCs, was the highest among the different experimental groups in Fig. 2A. In addition, the increase in the proliferative response of the F76 clone was associated with an increased production of IL-2 (from 1.5 ± 0.1 to 6.2 ± 0.3 U/ml) and IFN-γ (from 5.5 ± 0.27 to 11.4 ± 0.6 ng/ml).

Although we have previously demonstrated that the capacity of CD40 ligation or IL-6 to confer priming ability on peptide-loaded CD8+ DCs in a primary response in vivo is unlikely to involve the phenotypic conversion of CD8+ DCs into the other subset or the selective expansion of CD8− DCs (12, 13), we were concerned with the possibility that the CD8+ subset may mature into CD8− DCs upon CD40 ligation. However, cytofluorometric analysis of the CD8+ population after anti-CD40 treatment revealed that the percentage of CD8− DCs was virtually unchanged (>95%) and so was that of the CD8+ CD11c− fraction (<3%).

DCs not only stimulate T cells effectively but are also producers of cytokines that have important immune regulatory functions. It has been reported that CD4+ Th1 cells cultured with CD8− DCs are the main producers of IFN-γ and that CD8+ DCs in contrast produce very large amounts of IL-12 and IFN-α (16). To investigate whether a cytokine-mediated pathway may be at work in the defect in Th1 proliferation induced by CD8+ DCs, we measured the production of IL-12, IFN-γ, and IFN-α by CD8− and CD8+ DCs in the presence or the absence of CD40 ligation. The baseline production of IFN-α was below the detection limit of the assay (i.e., 2 IU/ml) and production remained undetectable after CD40 activation (data not shown). In addition, Fig. 2B shows that the baseline productions of IL-12 and IFN-γ were also limited. However, following CD40 activation, the levels of IL-12 and IFN-γ appeared to increase significantly in both DC subsets. Although the apparent lack of IFN-α production was somewhat unexpected, it has been reported that optimal release of IFN-α by CD8+ DC may require a combination of CpG and poly(I:C) (16).

Defective CD40L expression in Th1 cells cultured with CD8− DCs

The ability of CD40 activation to prime CD8+ DCs for effective support of Th1 cell growth suggested that defective expression of CD40L may be associated with the poor proliferative response of
the latter cells upon coculture with untreated CD8\(^+\) DCs. As a matter of fact, a crucial role in the activation and function of DCs is considered to be played by the CD40-CD40L interaction (19). CD40L is expressed on activated mature T cells but not on resting T cells. The expression of CD40L on activated T cells is transient and tightly regulated. However, the factors that contribute to the regulation of CD40L expression are not entirely known (30). We wanted to examine CD40L expression in Th1 cells cocultured with CD8\(^-\) vs CD8\(^+\) DCs in the presence of Ag peptide (Fig. 3). We found that CD40L expression could be clearly detected at 24 h of coculture of the Th1 cells with CD8\(^-\) DCs, and peak levels were reached at ~48 h. In contrast, the limited CD40L expression observed at 24 h of cell incubation with CD8\(^+\) DCs was totally abrogated at 48 h. When the same cocultures of Th1 and CD8\(^+\) or CD8\(^-\) DCs were examined for CD28 expression, we found that the percentages of CD28\(^+\) T cells incubated with CD8\(^+\) DCs were 45.3 at 3 h, 44.9 at 24 h, and 46.7 at 48 h; on incubation of the T cells with CD8\(^-\) DCs, the percentages were 46.1 (3 h), 44.5 (24 h), and 45.5 (48 h). Therefore, the poor proliferative response of Th1 cells to peptide-pulsed CD8\(^-\) DCs appeared to correlate with defective expression of CD40L on the former cells. In parallel experiments, we also found that the Th2 cells were induced to express high levels of CD40L by both CD8\(^-\) and CD8\(^+\) DCs. As an example, the 3-48-h expressions of the Th2 clone were 5.6/82.0% (for CD8\(^-\) DC coculture) and 4.9/80.4% (for CD8\(^+\) DC coculture).

**Increased CTLA-4 expression in Th1 cells cultured with CD8\(^-\) DCs**

CTLA-4 present on CD4\(^+\) T cells acts as a key negative modulator of immune responses by blocking CD28-dependent T cell activation (31). CTLA-4 is also involved in the induction of peripheral T cell tolerance in vivo (32). Therefore, CTLA-4 normally acts as a repressor of T cell activation. Recent evidence suggests that up-regulation of CTLA-4 levels may occur after anti-CD40L treatment, leading to attenuation of Th1 cell activation (22). It appeared therefore of interest to investigate CTLA-4 expression in Th1 cells cultured with CD8\(^-\) and CD8\(^+\) DCs. Although CTLA-4 expression is largely intracellular, TCR ligation induces rapid movement from endosomes to plasma membrane. Nonetheless, CTLA-4 is quickly endocytosed, which precludes significant accumulation on the surface. To examine regulation of the cycling of CTLA-4 between endosomal compartment and the cell surface, the Th1 cells were incubated for different times with either type of DCs before cytofluorometric analysis of expression of surface CTLA-4. Fig. 4A shows that a dramatic increase in CTLA-4 expression was observed at 24–48 h only in Th1 cells exposed to CD8\(^+\) DCs. When the intracellular levels of CTLA-4 were measured in permeabilized cells, we found that significant and comparable increases were observed in Th1 cells regardless of the DC subtype added to the coculture (Fig. 4B). Therefore, coculture of Th1 cells with CD8\(^+\) DCs appeared to increase the surface expression of CTLA-4 and to influence the rapid cycling between endosomal compartments and the cell surface. It is interesting to note that, in parallel experiments using Th2 cells, we found that the CD8\(^+\) DCs would induce a limited expression of surface CTLA-4 (data not shown).

**Blockade of CTLA-4 up-regulates CD40L expression and restores the proliferative response of the Th1 clone**

Blockade of CTLA-4 interactions using neutralizing mAb has been found to augment T cell proliferation in vitro (26, 31), to promote tumor rejection in vivo (33), and to prevent Ag-specific tolerance.

**FIGURE 3.** CD40 ligand expression in Th1 cells cultured with CD8\(^-\) or CD8\(^+\) DCs. Cultures were established using F76 cells and CD8\(^-\) or CD8\(^+\) DCs in the presence of Ag peptide. At 3, 24, or 48 h of coculture, gated CD3\(^+\) cells were examined for expression of CD40L by means of PE-conjugated MR1 mAb (bold lines). Control staining consisted of PE-conjugated hamster IgG (thin lines). Numbers within boxes indicate the percentage of CD3\(^+\) cells staining positively for CD40L. Data are from one experiment representative of four.

**FIGURE 4.** CTLA-4 expression in Th1 cells cultured with CD8\(^-\) or CD8\(^+\) DCs. Cultures were established using purified DC fractions and F76 cells in the presence of cognate peptide. Gated CD3\(^+\) cells were examined for expression of CTLA-4 at 3, 24, or 48 h of coculture. A. Surface staining with anti-CTLA-4-4-PE (bold lines) or control PE-conjugated hamster IgG (thin lines). Numbers within boxes indicate the percentage of CD3\(^+\) cells staining positively for CTLA-4. One experiment representative of three. B. Intracellular staining with anti-CTLA-4-PE or control Ab using permeabilized cells. Data are the means ± SD of three independent experiments.
We investigated whether the copresence of mAb to CTLA-4 would affect the ability of CD8⁺ DCs to sustain the growth of the Th1 clone. Cultures were established using CD8⁺ DCs and the Th1 clone F76 in the presence of the cognate peptide, with or without the anti-CTLA-4 4F10 mAb. Fig. 5A shows that the poor ability of the CD8⁺ DCs to sustain the growth of the Th1 cells was completely overcome by blockade of CTLA-4. When CD40L expression was examined in the Th1 cells subject to CTLA-4 blockade during coculture with CD8⁺ DCs, a remarkable increase in this expression was found to occur at ~24–48 h (Fig. 5B). On examining the production of IL-2 and IFN-γ by Th1 cell cultures treated with anti-CTLA-4 in the presence of CD8⁺ DCs, we found that blockade of CTLA-4 would result in increased production of IL-2 from 1.0 ± 0.1 to 4.6 ± 0.2 U/ml and IFN-γ from 5.1 ± 0.2 to 9.4 ± 0.4 ng/ml.

**Decreased CTLA-4 expression in Th1 cells cultured with CD40-activated CD8⁺ DCs**

In chronic experimental myasthenia gravis, the blockade of CD40L in vivo is known to affect both T cell effector and APC functions, with a reduction in B7-2, IL-12, and IFN-γ levels and increased expression of CTLA-4 (22). We wanted to examine the effect of CD40 activation in vitro in CD8⁺ DCs on the expression of CTLA-4 by Th1 cells. CD8⁺ DCs were subjected to CD40 cross-linking as illustrated above before coculture with Th1 cells in the presence of Ag peptide. Regulation of recycling of CTLA-4 between endosomal compartment and cell surface was investigated. Fig. 6A shows that considerable inhibition of cell surface expression of CTLA-4 was observed at 24–48 h in Th1 cells incubated with CD40-activated CD8⁺ DCs, thus opposing the dramatic increase in CTLA-4 observed in the absence of CD8⁺ DC treatment. In contrast, no major changes were found in the intracellular levels of CTLA-4 under comparable experimental conditions (Fig. 6B). Therefore, it appeared that CD40-mediated changes of CTLA-4 expression by T cells would occur in association with the increased Th1 cell proliferative response observed in Fig. 2.

**Discussion**

The interaction of CD4⁺ T cells with DCs is known to be a critical step in the initiation of acquired immunity and can result in a

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**FIGURE 5.** Blockade of CTLA-4 increases CD40L expression and proliferation in Th1 cells. Cultures were established using peptide-pulsed CD8⁺ DCs and F76 cells in the presence of anti-CTLA-4 4F10 mAb. A, Proliferation was measured at 48 h in Th1 cells exposed to different concentrations of CD8⁺ DCs (indicated) in the presence of anti-CTLA-4 mAb, control Ig, or medium alone. Data are the means ± SE in one experiment representative of three. B, Surface expression of CTLA-4 using anti-CTLA-4-PE (bold lines) or hamster IgG-PE (thin lines) in intact cells. Numbers within boxes indicate the percentage of CD3⁺ cells staining positively for CTLA-4. One experiment representative of three.

**FIGURE 6.** CTLA-4 expression in Th1 cells cultured with CD40-activated CD8⁺ DCs. Cultures were established using F76 cells and peptide-pulsed CD8⁺ DCs, either as such or after CD40 cross-linking. Gated CD3⁺ cells were examined for expression of CTLA-4 at 3, 24, or 48 h of coculture. A, Surface expression of CTLA-4 using anti-CTLA-4-PE (bold lines) or hamster IgG-PE (thin lines) in intact cells. Numbers within boxes indicate the percentage of CD3⁺ cells staining positively for CTLA-4. B, Expression of intracellular CTLA-4 in permeabilized cells reacted with anti-CTLA-4-PE or control Ab. Data (percentage of positive cells) are the means ± SD of three independent experiments.
change in the functional state of both the CD4+ T cell and the DC. A naive CD4+ T cell becomes primed to participate more efficiently in cognate interaction with other cells of the immune response and the DC becomes more efficient at interacting with CD8+ T cells (35–37). In the mouse, there are different subclasses of DCs whose interactions with T cells have different outcomes (38). The myeloid-related CD8+ DCs are efficient APCs capable of activating naive T cells to proliferate and produce cytokines, whereas the CD8- DCs are less potent despite their mature phenotype and equivalent expression of MHC and costimulatory molecules (27, 39).

CD8+ and CD8- DCs differ in their ability to induce proliferative responses in both primary CD4+ and primary CD8+ T cells (14, 17). Recent evidence using freshly harvested secondary T cells indicates that CD8- DCs can activate Ag-primed CD4+ T cells and stimulate proliferation. In contrast, interaction with CD8+ DCs, though able to induce clustering of the T cells, results in only poor and sometimes barely significant levels of both cytokine production and proliferation (18). Immature DCs may exert immunoregulatory effects also through interaction with T regulatory cells (40). However, a recent study on the regulatory activity of immature and mature CD8+ DCs has indicated that neither immune deviation nor induction of regulatory cells may be a significant contributory factor to the modulation in vivo of peripheral T cell function (41).

CD8- DCs were initially described as the major producer of IL-12 p70 (42–45) and were reported to induce predominantly Th1 responses, whereas CD8- DCs drive Th2 or mixed Th1/Th2 responses (44, 46). However, recently, the capacity of all CD11c+ DCs to produce IL-12 p70, and thus also their ability to induce Th1 responses, has been shown to vary with Ag stimulus (47, 48). In the present study, we made use of highly polarized T cell clones with the same Ag specificity to comparatively analyze the ability of CD8- and CD8+ DCs to stimulate the proliferative response of Th1 vs Th2 cells in the presence of specific Ag.

By using an Ag peptide that is presented differentially by CD8- and CD8+ DCs in a primary response in vivo (8–13), we made a series of observations that may be relevant to a better understanding of the reciprocal control of Th cell and DC function during the course of a secondary response. We found that defective ability to sustain T cell proliferation was selectively shown by CD8- DCs cultured with Th1 cells (Fig. 1). During the coculture, the latter cells were induced to express considerable amounts of CTLA-4 (Fig. 4), which event was associated with impaired ability to express the CD40L (Fig. 3) and unchanged expression of CD28. Poor expression of CD40L by the T cells likely contributed to suboptimal interaction between DCs and Th1 cells, such that CD40 activation on the DCs fully restored their ability to sustain the growth of the Th1 clone (Fig. 2). Reciprocal regulation appeared to occur between CTLA-4 and CD40L expression, because CTLA-4 neutralization led to increased proliferation and up-regulation of CD40L expression (Fig. 5), and CD40 activation on DCs down-regulated the expression of CTLA-4 (Fig. 6) and up-regulated that of CD40L (data not shown). It is interesting to note that, in line with previous observations (49), CD28 was expressed similarly by the Th1 and Th2 clones used in this study (data not shown).

CTLA-4 is expressed on T cells after activation and shares homology with the CD28 costimulatory receptor. In contrast to CD28, CTLA-4 is considered to be a negative regulator of T cell activation. Cross-linking of CTLA-4 during activation of peripheral T cells reduces IL-2 production and arrests T cells in G1 (23–25). In differentiated T cells, CTLA-4 can function to suppress the production of cytokines produced by both Th1 and Th2 cells (49). Several mechanisms have been proposed to explain the inhibitory activity of CTLA-4, including competition for ligand access to CD28, delivery of a signal that antagonizes a CD28 signal, and delivery of a signal that antagonizes a TCR-mediated event (26). Recent evidence indicates that CTLA-4 up-regulation may represent a likely mechanism whereby signaling through CD45 in T cells mediates tolerogenic effects (50). Up-regulation of CTLA-4 has also been shown to result from blockade of CD40L in T cells (22).

Our observation that CTLA-4 is up-regulated in Th1 cells exposed to peptide-pulsed CD8+ DCs suggests that these cells may directly regulate the expression of surface CTLA-4 and influence the rapid cycling between endosomal compartments and the cell surface. Although the regulation of CTLA-4 expression is complex and occurs at multiple levels (23, 24), the recent demonstration of a link between CD45 and CTLA-4 that depends on calcineurin-mediated signaling (50) may underscore a role for the activation of such a mechanism by peptide-pulsed CD8+ DCs. Interestingly in this regard is the observation that CD45 may be differentially expressed on Th1 and Th2 cells (51). Although the data of the present work were generated using a single Th1 clone and a single Th2 clone, we have very recently observed a similar pattern of effects using Th1 and Th2 clones generated in our laboratory to NRP, a synthetic peptide mimotope recognized by diabetogenic T cells in the nonobese diabetic mouse (Ref. 13 and data not shown). In addition, results similar to ours, of a differential effect of CD8- and CD8+ DCs, have been reported for the stimulation of secondary CD4+ T cells specific for influenza virus hemagglutinin (18).

Whatever the mechanisms whereby surface CTLA-4 becomes up-regulated in Th1 cells cultured with CD8- DCs, one major observation in the present study is that this event results in impaired expression of the CD40L, such that blocking of CTLA-4 by means of specific Ab will result in up-regulation of CD40L and restore proliferative ability. Again, several mechanisms can be proposed to explain the inhibitory activity of CTLA-4 on CD40L expression, including inhibition of CD28-mediated effects and generation of signals that antagonize or abort TCR-driven events.

Triggering of DCs in vivo through CD40 is a powerful activa-
tion stimulus, causing these cells to express the full array of Ag-presenting/costimulatory molecules (30, 20). Moreover, injection of CD40-modulated DCs restores Ag-specific CTL responses in CD4+ T cell-depleted mice (36). These data indicate that the function of CD4+ Th cells is mediated through CD40-dependent activation of APCs. Recent evidence suggests that the CD40-CD40L pair can act as a switch in vivo, determining whether naive peripheral CTL are primed or tolerized (52) and accounting for the ability of CD40 ligation to convert tumor-specific CD4+ T cell tolerance into T cell priming (53). Of interest in this regard may be our current observation that CD40 activation of CD8+ DCs will result in impaired expression of CTLA-4 by the Th1 clone cultured with those cells. Although the underlying mechanism remains to be determined, it is possible that an optimally conditioned CD8+ DCs may affect the rapid cycling of CTLA-4 between endosomal compartments and cell surface so as to limit the surface expression of CTLA-4.

In conclusion, the poor ability of CD8+ DCs to support the growth of a Th1 clone can be overcome by CD40 activation, a maneuver that apparently obviates the poor expression of CD40L by the Th1 cells cultured with CD8+ DCs. One possible mechanism via which the T cells are prevented from expressing significant amounts of CD40L may be represented by increased expression of surface CTLA-4, whose neutralization has effects comparable to those of CD40 activation. These data may be among the first to report on mutual regulation between CTLA-4 and...
which distinct DC subsets interact with Ag-primed CD4+ T cells.

Acknowledgments

We thank Dr. Maria Ferrantini for assistance with the IFN-α biological assay.

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