CTL-4 Regulates Expansion and Differentiation of Th1 Cells Following Induction of Peripheral T Cell Tolerance

Todd N. Eagar, Danielle M. Turley, Josette Padilla, Nitin J. Karandikar, Litjen Tan, Jeffrey A. Bluestone and Stephen D. Miller

http://www.jimmunol.org/content/172/12/7442

References
This article cites 29 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/172/12/7442.full#ref-list-1

Subscription
Information about subscribing to J Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
CTL-4 Regulates Expansion and Differentiation of Th1 Cells Following Induction of Peripheral T Cell Tolerance

Todd N. Eagar,*† Danielle M. Turley,* Josette Padilla,* Nitin J. Karandikar,²* Litjen Tan,* Jeffrey A. Bluestone,¹ and Stephen D. Miller³*

Intravenous treatment with Ag (peptide)-coupled, ethylene carbodiimide-fixed syngeneic splenocytes (Ag-SP) is a powerful method to induce anergy in vitro and peripheral T cell tolerance in vivo. In this study, we examined the effects of Ag-SP administration on T cell activity ex vivo and in vivo using OVA-specific DO11.10 TCR transgenic T cells. Although treatment with OVA323–339-SP resulted in a strong inhibition of peptide-specific T cell recall responses in vitro, examination of the immediate effects of Ag-SP treatment on T cells in vivo demonstrated that tolerogen injection resulted in rapid T cell activation and proliferation. Although there was an increase in the number of OVA-specific DO11.10 T cells detected in the lymphoid organs, these previously tolerized T cells were strongly inhibited in mounting proliferative or inflammatory responses upon rechallenge in vivo with peptide in CFA. This unresponsiveness was reversible by treatment with anti-CTL-4 mAb. These results are consistent with the hypothesis that Ag-SP injection induces a state of T cell anergy that is maintained by CTL-4 engagement. The Journal of Immunology, 2004, 172: 7442–7450.

Antigen-specific tolerance is a method by which Ag administration results in future decreased T cell activity to a given Ag. The ability to establish Ag-specific T cell unresponsiveness is a much sought after goal for the treatment of autoimmune and other inflammatory diseases (1). Tolerance relies upon Ag administration in a context different from immunization. It has been found, for example, that Ag given orally, by i.v. injection or in the context of fixed APC, can induce Ag-specific tolerance. Three basic mechanisms have been described to account for the loss of T cell responses following tolerance: clonal deletion, anergy induction, and the production of regulatory T cells (reviewed in Ref. 2). Clonal deletion leads to the loss of Ag-specific T cells by cell death. Several molecules have been identified as players in this process, including the apoptosis mediators Fas, Fas ligand, and others (3). T cell anergy is a specific state of the T cell in which they are incapable of responding to normally immunogenic signals. Specifically, induction of anergy is thought to result from Ag encounter in the absence of sufficient costimulatory signals (4) and anergic T cells produce significantly lower amounts of IL-2 and have defects in downstream signaling pathways upon TCR ligation (5, 6). Several lines of evidence now point to a role for CTLA-4-mediated signals in regulating some forms of anergy (7–11). A third potential mechanism of tolerance is the induction of T cells with a regulatory phenotype that can be induced to produce IL-10, TGF-β, or other cytokines that negatively regulate neighboring T cell responses (12). Recently, it has been identified that peripheral tolerance models may induce the function of a CD4⁺CD25⁺ suppressor cell population (13, 14).

Ethylene carbodiimide (ECDI)⁴-fixed APC were used in many of the studies that described the induction of T cell clonal anergy both in vivo and in vitro (15, 16). These studies helped to define the two-signal model of T cell activation and the requirement for costimulatory interactions in this process. Intravenous administration of Ag (peptide)-coupled, ECDI-fixed splenocytes (Ag-SP) has been proven to be an effective method of inducing T cell unresponsiveness in vivo (15–17). Previous work by our laboratory has described the efficacious use of myelin epitope-coupled splenocytes for regulating the induction and progression of experimental autoimmune encephalomyelitis (EAE), a murine Th1-mediated model of the CNS inflammatory demyelinating disease multiple sclerosis (18–20). Furthermore, tolerance induced using Ag-SP relies upon the inhibitory function of CTLA-4, which we have shown is required to maintain the unresponsive state of the tolerized T cells upon antigenic stimulation under inflammatory conditions (7).

Using an adoptive transfer model using a traceable population of CFSE-labeled OVA323–339-specific DO11.10 transgenic T cells, we show that while in vivo and in vitro peptide-specific T cell responses are significantly reduced following i.v. OVA323-SP treatment, the tolerizing regimen has the paradoxical effect of stimulating activation and proliferation of naive Ag-specific T cells. Previously tolerized T cells, upon secondary encounter with Ag under inflammatory conditions, i.e., in the context of s.c. immunization with peptide in CFA, are inhibited from functioning at the level of T cell proliferation and the ability to perform effector inflammatory functions. Consistent with our previous findings, the decrease in T cell responses was controlled by CTLA-4.

¹Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Northwestern University Medical School, Chicago, IL 60611; and ²Diabetes Center, University of California, San Francisco, CA 94143

Received for publication January 12, 2004. Accepted for publication April 2, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by U.S. Public Health Service National Institutes of Health Research Grants NS34819, NS30871, NS26543, AI35294, CA40216, and AI35225.

2 Current address: Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75235.

3 Address correspondence and reprint requests to Dr. Stephen D. Miller, Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611. E-mail address: s-d-miller@nwu.edu

4 Abbreviations used in this paper: ECDI, ethylene carbodiimide; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; Ag-SP, Ag-coupled splenocyte.
Materials and Methods

Mice

BALB/c mice, 6- to 7-wk-old, were purchased from The Jackson Laboratory (Bar Harbor, ME). DO11.10 TCR transgenic mice were bred at Northwestern University. All mice were housed under specific pathogen-free conditions (viral Ag-free) in the Northwestern animal care facility with standard laboratory food and water ad libitum. All mouse work was done in accordance with Animal Care Use Committee protocols.

Peptides

Synthetic peptides of chicken OVA323–339 (ISQAVHAAHAEINEAGR) and proteolipid protein peptide PLP139–151 (HSLGKWLGHPDKF) were purchased from Peptides International (Louisville, KY).

Induction of peripheral tolerance and immunization

Tolerance was induced using i.v. injections of chemically treated Ag-coupled syngeneic splenocytes, as described previously (15, 16). A modification of the standard Ag-coupled splenocyte protocol was used in which mice are injected with 5 × 10^6 fixed splenocytes and receive a treatment of 100 μg of LPS (Sigma-Aldrich, St. Louis, MO). This treatment is immunogenic and is a better approximation of the kinetics for T cell activation as Ag-coupled splenocyte treatment than immunization with OVA323–339/CFA injected s.c. (T. Eagar, unpublished observations). Mice were immunized over three spots on the flank with 100 μl of CFA containing 100 μg of OVA323–339 or 50 μg of PLP139–151 and 200 μg of Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI), as previously described (21).

Abs and recombinant protein treatments

Hamster control Ig (Cappel Research Products, Durham, NC), anti-CTLA-4 mAb (UC10-4F10), and CTLA-4 Ig were used, as indicated (22). Anti-CTLA-4 F(ab) were produced as previously described (22).

Delayed-type hypersensitivity (DTH) responses

DTH responses were quantitated using a 24-h ear-swelling assay, as previously described (23). Results are expressed in units of 10^{-1} inches ± SEM. Basically, the mice are injected with 10 μl of saline containing 10 μg of OVA323–339 peptide. This protocol was modified in the experiments for Fig. 6 to include 2 μg of hamster Ig or anti-CTLA-4 in the challenge solution.

Flow cytometry

Abs specific for CD4, CD25, CD45RB, CD90, CD95, and CD152 were purchased from BD Pharmingen (San Diego, CA). Anti-clonotype KJ1-26 was purchased from R&D Systems (Minneapolis, MN). Isolated spleen and lymph node cells were prepared for flow cytometry by incubating cells with each Ab, followed by PBS washes and fixation with 4% paraformaldehyde (Sigma-Aldrich). For intracellular protein analysis, cells were permeabilized with 0.1% Saponin. Data collection and analysis were performed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using forward scatter, side scatter, and other (indicated) gates.

CFSE proliferation assays

T cells were incubated in PBS containing CFSE (Molecular Probes, Eugene, OR) at a concentration of 2.5 μM. Following incubation, fluorescence was quenched by further incubation in serum-containing medium. Dilution of CFSE, indicative of cellular division, was determined by flow cytometry. Cell numbers at a given cell generation were determined by gating on each generation and calculating the percentage of cells in each generation as compared with the total number of transgenic T cells.

Statistical analyses

Comparisons of DTH responses between any two groups of mice were analyzed by Student’s t test.

Results

Intravenous treatment with Ag-SP inhibits T cell recall responses without inducing clonal deletion

Experiments designed to examine the effects of Ag-SP tolerance on T cell activity rely on two administrations of Ag. The first treatment, the tolerance induction step, consists of the i.v. injection of Ag-SP and is thought to result in direct interaction between the peptide-pulsed, ECDI-fixed APC, and the T cell. The second encounter is at the time of immunization with peptide emulsified in CFA. These two encounters can be separated by as few as 24 h or more than 3 wk and still be efficacious in inducing Ag-specific unresponsiveness (15) (T. Eagar, unpublished observations). To gain a further understanding of the mechanistic basis of Ag-SP tolerance, we used a model in which tolerance could be assessed in vivo in an Ag-specific manner in a traceable specific T cell population (OVA323–339/L-A^4-restricted T cells from DO11.10 TCR transgenic mice) (24).

We first examined the effects of Ag-SP on T cell proliferation in the DO11.10 transfer model. BALB/c recipients of transgenic T cells were left untreated or were injected with Ag-SP that had been coupled to either PLP139–151 (Sham-SP) or OVA323–339 (OVA323-SP). Five days later (day 0), the mice were immunized with OVA323–339/CFA, and proliferation assays were performed on day +5. Peptide-induced proliferation of lymph node cells from OVA323-SP-injected mice was dramatically reduced in comparison with responses in Sham-SP-treated mice (Fig. 1A). To determine whether this unresponsive state was due to deletion of peptide-specific T cells, naïve BALB/c mice were injected with CD4^+ DO11.10 T cells, and 24 h following cell transfer (day −5), the recipient mice were treated with Sham-SP, OVA323-SP, or OVA323–339/CFA. On day 0, the numbers of KJ1-26^+ T cells were quantitated by flow cytometry. Also on day 0, a group of Sham-SP- and OVA323-SP-treated mice was immunized with OVA323–339/CFA, and transgenic cell numbers were quantitated an additional 5 days later. Fig. 1B shows the effects of each treatment on total transgenic T cell numbers from pooled spleen or lymph node nodes from three mice per group, and the data are expressed as the total number of cells per organ per mouse. As compared with the Sham-SP-treated controls, transgenic T cell recipients treated i.v. with OVA323-SP or primed s.c. with OVA323–339/CFA had increased numbers of transgenic T cells both in the lymph nodes and spleens (Fig. 1B). Mice that had received dual treatments of OVA323-SP and OVA323–339/CFA retained similar numbers of transgenic cells and were not deleted. These results indicate that the major effect of Ag-SP was the induction of T cell anergy in vivo rather than deletion.

Normalization of T cell proliferative responses from the bulk lymph node cells in Fig. 1B strengthens the hypothesis of anergy as the primary mechanism of unresponsiveness. Bulk lymph node cells from the mice in Fig. 1B were stimulated with the indicated concentrations of OVA323–339. Ninety-six-hour proliferative responses were determined by [3H]TdR incorporation and were multiplied by the percentage of KJ1-26^+ T cells present in the total lymph node cell population. The normalized proliferation counts demonstrate that the transgenic T cells from OVA323-SP-tolerized mice proliferate poorly compared with cells from mice treated with Sham-SP (Fig. 1C). This effect was also observed in mice that received Ag-SP treatment and were later immunized with OVA323–339/CFA. These results support the hypothesis that following Ag-SP treatment, Ag-specific T cells are present, yet are unresponsive to additional stimulation.

The direct effects of in vivo Ag-SP encounter with naive Ag-specific T cells on the expression of activation markers were also examined (Fig. 1D). The levels of CD25, CD62L, and CD69 activation makers were measured by FACS on transferred DO11.10 cells 18 h after i.v. treatment of intact DO11.10 mice with either OVA323-SP or Sham-SP. The immediate effect of Ag-SP treatment was the activation of the Ag-specific T cells, as indicated by the up-regulation of CD25 and CD69 and the down-regulation of CD62L on the T cells. The peak change in activation marker expression occurred before 72 h (data not shown).
Effect of Ag-SP on peptide-specific T cell responses at the tolerogenic encounter

The fact that increased numbers of OVA323–339 Tg T cells were observed following OVA323-SP administration (Fig. 1) suggested that encounter with the fixed APC stimulated proliferation of the transgenic T cells. To directly test this possibility, CD4⁺ DO11.10 transgenic T cells that had been labeled with CFSE were transferred into naive recipients that were left untreated (naive) or were injected with Sham-SP, OVA323-SP, or OVA323–339/CFA 2 days later. Seventy-two hours posttreatment, splenocytes and lymph node cells were isolated and analyzed for CFSE dilution and expression of activation markers, CD45RB and CD95, by flow cytometry. Sham-SP treatment stimulated neither the activation nor the proliferation of transgenic T cells in vivo, similar to the pattern observed in untreated naive recipients (Fig. 2). In contrast, surprisingly, both the tolerogenic (i.e., OVA323-SP) and immunogenic (i.e., OVA323–339/CFA) encounter with OVA peptide stimulated high levels of proliferation and T cell activation in vivo. Interestingly, the T cells that had divided, from both the tolerized and immunized mice, expressed high levels of total cellular CTLA-4 (data not shown), once again consistent with activation.

Ag-SP tolerance inhibits in vivo T cell proliferative and DTH responses in a CTLA-4-dependent manner

The initial activation and in vivo proliferative responses of peptide-specific T cells following tolerance (i.e., OVA323-SP) and immunization (i.e., OVA323–339/CFA) are remarkably similar (Fig. 2), yet T cells exposed to OVA323-SP are unable to respond to secondary Ag encounter (Fig. 1). To examine the responses of tolerized T cells to secondary Ag encounter in vivo, intact DO11.10 TCR transgenic mice were treated with Sham-SP, OVA323-SP (tolerized), or OVA323–339-SP + LPS (immunized) (Fig. 3A). Three days following treatment, CD4⁺ T cells from the transgenic mice were isolated, labeled with CFSE, and transferred into naive BALB/c recipients. An additional 2 days later (day 0), the recipient mice were immunized with PLP139–151 (irrelevant control immunization) or OVA323–339, and in vivo DTH and proliferative responses were determined from three mice per treatment group. On day 5 following immunization, OVA323–339-specific DTH responses were determined by 24-h ear-swelling responses following intradermal challenge with 10 μg of soluble OVA323–339 peptide. As shown in Fig. 3B, the recipients of Sham-SP- or OVA323-SP + LPS-treated cells showed strong OVA323–339 DTH responses following immunization with
OVA323–339/CFA, but not with PLP139–151/CFA. In contrast, recipients of OVA323-SP-treated Tg cells failed to produce a strong DTH to OVA323–339 regardless of the priming Ag. In vivo proliferative responses were determined by flow cytometry on day 3 postimmunization. As demonstrated in Fig. 3C, splenic T cells did not proliferate in response to immunization with the irrelevant PLP139–151 peptide. Following immunization with OVA323–339/CFA, however, differences were detected in the ability of the transgenic T cells to respond by proliferation. T cells that had been previously treated with Sham-SP or immunized with OVA323–339/CFA + LPS strongly proliferated following immunization, achieving seven to eight rounds of division. However, T cells that had been tolerized by prior treatment with OVA323-SP were significantly inhibited in their ability to proliferate in vivo following peptide immunization with few cells dividing more than twice. Thus, OVA323-SP tolerance blocks both proliferation and effector differentiation of peptide-specific Th1 cells in response to secondary immunization in vivo. Activation and proliferation of naive T cells require both TCR and costimulatory signals. Tolerance with Ag-coupled splenocytes uses bulk splenocytes from naive mice that are low expressers of many costimulatory molecules (data not shown). In addition, ECDI treatment has been shown to block the normal delivery of costimulatory signals, resulting in T cell anergy in vitro (16). To determine whether the proliferation of naive T cells induced by Ag-SP associated with tolerance induction requires B7-mediated costimulation or whether the up-regulation of CTLA-4 expression has functional consequences in the tolerized or immunized T cells, we examined the effects of blocking the interaction between B7 and its ligands, CD28 and CTLA-4, at the time of s.c. immunization with OVA323–339/CFA vs i.v. treatment with OVA323-SP. Naive BALB/c mice received 5 × 10⁶ CFSE-labeled, CD4⁺ DO11.10 transgenic T cells. Two days later, the mice were given the first of three daily treatments with CTLA-4 Ig (75 μg/treatment), anti-CTLA-4 (50 μg/treatment), anti-CTLA-4 F(ab) (50 μg/treatment), or control hamster Ig (50 μg/treatment). The treatments were given flanking the day of OVA323–339/CFA immunization or injection with Sham-SP or OVA323-SP. At 72 h postimmunization/injection, in vivo T cell proliferative responses were determined by flow cytometry. The data plotted in Fig. 4 are expressed as the percentage of total CFSE-labeled T cells that were found at each of seven discernible T cell generations (Fig. 4, A and B). Regression analysis demonstrates the minimum percentage of input transgenic T cells that are required to enter the cell cycle to produce the number of cells that had proliferated (Fig. 4, C and D).

Immunization with OVA323–339/CFA stimulated ~14% of the input T cells to enter the cell cycle, leading to up to seven rounds of division (Fig. 4, A and C). Treatment with CTLA-4 Ig significantly inhibited proliferation, as assessed by both the percentage of cells undergoing mitosis (~6%, a 60% reduction) and the maximum number of cell divisions achieved, while treatment with anti-CTLA-4 increased the number of cells undergoing mitosis, an effect that was amplified by injection of the F(ab) (Fig. 4C). Interestingly, anti-CTLA-4 administration did not result in an increased accumulation of T cells that had divided, indicating that these T cells were lost from the spleen by increased trafficking and/or apoptosis (Fig. 4C). No proliferation was observed following Sham-SP treatment, and is therefore not shown. However, i.v. OVA323-SP injection stimulated 14.7% of transgenic T cells to undergo proliferation, resulting in up to seven rounds of division (Fig. 4, D and B, respectively). Treatment with CTLA-4 Ig also reduced the percentage of cells entering the cell cycle in response to OVA323-SP as compared with controls and slowed the progression of the most rapidly dividing population of transgenic T cells. As opposed to the dramatic effects seen in peptide-immunized mice, treatment with intact or F(ab) of anti-CTLA-4 resulted in a slight decrease in percentage of cells that entered the cell cycle, but did not significantly alter the extent of T cell proliferation in response to i.v. OVA323-SP injection. These results indicate that blocking CD28 with CTLA-4 Ig inhibits T cell activation in both tolerogenic and immunogenic conditions, while anti-CTLA-4 has opposite effects on primed vs tolerized T cells. To assess the role of CTLA-4 in delaying or preventing the proliferation of tolerized T cells to a subsequent immunogenic stimulus, cells from DO11.10 transgenic mice that had been treated with Sham-SP, OVA323-SP, or OVA323-SP + LPS were isolated, labeled with CFSE, and transferred into naive BALB/c mice. Two days later, on day 0, the mice were primed with OVA323–339/CFA. Surrounding the immunization, on days −1, 0, +1, and +2, the mice were treated with hamster Ig or anti-CTLA-4. OVA323–339-specific DTH responses were determined 7 days later. As shown in Fig. 5B, T cells from OVA323-SP-treated mice developed minimal DTH responses as compared with controls. Treatment with anti-CTLA-4, however, restored the level of DTH responses to Sham-SP-treated control levels. This result mirrors that which we have reported in the PLP139–151 model of EAE in the SJL mouse strain (7). To assess the effect of CTLA-4...
on T cell proliferation, flow cytometry was performed on splenocytes from the BALB/c recipients of OVA323-SP T cells that had been treated with hamster Ig or anti-CTLA-4. Anti-CTLA-4 increased both the number of cells that divided and the number of divisions (Fig. 5C). For comparison, the proliferative response of untreated T cells is shown. Further analysis demonstrated that the effect of anti-CTLA-4 was the result of a 60% increase in cells entering the cell cycle over T cells from OVA323-SP + control Ig-treated mice (Fig. 5D). A similar enhancement of proliferation was obtained when lymph node cells were examined for proliferation in anti-CTLA-4-treated recipients of tolerized T cells (data not shown).

**CTLA-4 regulates effector function of Th1 cells mediating DTH**

Our data indicate that Ag-SP tolerance results in the CTLA-4-mediated inhibition of both T cell proliferation in vivo and the effector function of T cells mediating DTH responses (Fig. 5). Multiple steps are involved in developing DTH responses. These steps include T cell activation, proliferation, differentiation, trafficking, and ability to activate macrophages via production of inflammatory chemokines and cytokines. Tolerance, and therefore CTLA-4, could thus function at multiple levels to inhibit a DTH response. Exposure of naive T cells to Ag-SP leads to significant activation at the tolerogenic encounter, but significantly retards T cell activation at the secondary immunogenic encounter. It is not clear at this point whether the T cells have differentiated sufficiently to carry out Th1 effector function. To address the ability of tolerized T cells to traffic to the site of Ag challenge, BALB/c mice were injected with CD4+ DO11.10 TCR transgenic T cells. One day following cell transfer, the mice were treated with Sham-SP or OVA323-SP. Five days later, mice were immunized with PLP139–151/CFA or OVA323–339/CFA (day 0). At day +5, OVA323–339-specific DTH responses were elicited. At 24-h postear challenge, the ears were prepared for immunohistochemical analysis. KJ1-26+ T cells were not detected in the ears of mice immunized with an irrelevant Ag (PLP139–151) (data not shown). Surprisingly, KJ1-26+ T cells were found in the ears of both the Sham-SP (Fig. 6A) and OVA323-SP (Fig. 6B) treated mice that had been immunized with OVA323–339/CFA. This suggests that the absence of DTH that is seen following tolerance is not due to the absence of Ag-specific T cells, but possibly to inhibition of effector function of those T cells in the peripheral tissues. The finding that Ag-specific T cells can migrate to the site of DTH challenge provides a method to examine the effects of CTLA-4 on regulating terminal effector function. BALB/c mice were thus injected with 5 x 10^6 CD4+ DO11.10 TCR transgenic T cells from donors that had been treated with Sham-SP, OVA323-SP, or OVA323-SP + LPS. Two days later, mice were immunized with OVA323–339/CFA (day 0). The normal DTH protocol was modified to include the injection of Ab into the ear at the time of challenge. Mice from each group received a 10 µl injection containing 10 µg of OVA323–339 peptide and 2 µg of either hamster control Ig (in the left ear) (□) or anti-CTLA-4 (in the right ear) (□) on day +5. Twenty-four hours following challenge, net ear swelling was quantitated. As shown in Fig. 6C, local administration of anti-CTLA-4 into the ear enhanced the DTH responses of all groups. The effect of anti-CTLA-4 injection was most dramatic in recipient mice receiving OVA323-SP-tolerized T cells, as DTH
responses were restored to a level comparable to the anti-CTLA-4-treated controls.

**Discussion**

The i.v. administration of peptide-pulsed, ECDI-fixed splenocytes (Ag-SP) is a powerful and Ag-specific method of in vivo tolerance induction. This method of tolerance induction has been shown to be able to not only prevent the onset of relapsing EAE, but to be an effective treatment for ameliorating the progression of established disease (19, 20, 25, 26). Due to the possible therapeutic potential of this tolerance protocol for treatment of human autoimmune diseases, it is critical to gain a thorough understanding of the cellular and molecular mechanisms of tolerance induction and maintenance in both naive and memory T cells. Crucial for the interpretation of these studies is the knowledge of the location of where Ag is being presented to the autoreactive T cells and in what context.

In this study, we describe the cellular mechanisms of tolerance induction by the i.v. injection of Ag-coupled splenocytes. Current and previous data (7) clearly show that treatment with Ag-SP induces a dramatic reduction in recall Ag-specific in vivo proliferative and DTH responses and in vitro proliferative and cytokine responses. Interestingly, it was found that the direct consequence of in vivo interaction of Ag-SP with naive T cells was activation and a rapid and net increase in T cell numbers in the spleen and lymph nodes of treated mice (Fig. 2). The increase in Ag-specific T cells following Ag-SP treatment indicates that the major effect of Ag-SP-induced tolerance is not the induction of clonal deletion. Comparing the proliferative responses on an individual transgenic cell basis suggests that the specific induction of unresponsiveness is the result of anergy induction. These experiments cannot, however, rule out the possibility that T cell deletion may occur at later time points following tolerance induction.

Further examination of the immediate effects of Ag-SP showed that the i.v. injection of the tolerogen was a strong stimulus for T cell activation (Fig. 1D) and the induction of cellular proliferation in vivo (Fig. 2). T cell activation following tolerance induction has been described in other models (8, 11, 27). The fact that the overwhelming majority of Ag-specific T cells become activated following tolerance induction suggests that activation may be crucial for the establishment of the unresponsive state; however, at this point it is not clear whether proliferation is required for tolerance induction. Additional experiments demonstrated that although CD25 was up-regulated following Ag-SP treatment, the expression of the regulatory T cell marker was not maintained for >72 h after tolerance induction (data not shown). Moreover, depletion of
CD25+ cells before Ag-SP tolerance does not inhibit the induction of specific T cell unresponsiveness (A. P. Kohn and S. D. Miller, unpublished observations). This suggests that tolerance is not the result of eliciting the function of an extant CD4+ CD25+ regulatory T cell population.

Despite the similar effects of immunization and tolerance induction with regard to activation and proliferation of naive T cells, these two treatments differ dramatically in their response to treatment with costimulatory antagonists. Following immunization, the proliferation of transgenic T cells was enhanced by treatment with anti-CTLA-4 and inhibited by CTLA-4 Ig. In Ag-SP-treated mice, however, proliferation was reduced by both CTLA-4 Ig and anti-CTLA-4 injection (Fig. 4). This may be explained as a result of different APC involved in the activation of the transgenic T cells following encounter with immunogenic vs tolerogenic forms of Ag. Following immunization, T cells would encounter Ag in the context of a dendritic cell or other professional APC that has been activated by innate immune stimuli provided by components of the CFA. Tolerance, in contrast, relies on the injection of fixed splenocytes from a naive donor mouse and does not induce an increase in in situ B7 expression. In fact, it appears that ECDI fixation leads to the apoptotic death of the splenic APC and most likely does not induce potent innate immune signals upon i.v. administration (D. M. Turley, T. N. Eagar, and S. D. Miller, unpublished observations). Despite the fact that CTLA-4 Ig blocked T cell proliferation following in vivo encounter with Ag-SP, our previous results (7) showed that in vivo administration of CTLA-4 Ig, which blocks both B7/CD28 and B7/CTLA-4 signals (28), surrounding the time of Ag-SP administration, did not alter the ability of peptide-coupled splenocytes to induce tolerance as measured by peptide-specific DTH. However, it should be noted that CTLA-4 Ig has been reported to prevent short-term T cell tolerance induced by i.p. administration of soluble peptide in IFA (8), thus indicating a difference between the two models of tolerance induction and perhaps reflecting an important role for immune deviation to Th2 responses in the latter tolerance model (29).

The responses most altered following Ag-SP therapy were seen during the secondary Ag encounter when T cells initially exposed to Ag-SP were restimulated in the context of the proinflammatory environment provided by immunization with peptide in CFA. T cells from tolerized mice were unable to elicit DTH responses (Fig. 3B), and previously tolerized splenic T cells demonstrated aborted proliferative responses both in vitro (Fig. 1) and in vivo (Fig. 3C) following immunization. Previously tolerized transgenic T cells from the spleen were inhibited in the early stages of activation, as they did not show an increase in size and complexity as determined
by forward scatter on the flow cytometric analysis (data not shown). This led us to hypothesize that CTLA-4 was directly regulating T cell activity following tolerance.

The effect of anti-CTLA-4 treatment on reversing the maintenance phase of tolerance appears to act at two levels. As we have previously demonstrated in the PLP139–151-coupled splenocyte tolerance model (7), effective reversal of tolerance was achieved when Ab was given at the time of immunization (Fig. 5B) or during autoimmune inflammation. Treatment with anti-CTLA-4 at the time of Ag-SP injection or between Ag-SP treatment and immunization did not restore DTH responses. This suggests that blocking CTLA-4 can restore activity of tolerized T cells when Ag is present. Blocking CTLA-4 at the time of immunization enhances the overall percentage of cells entering the cell cycle and extent of proliferation achieved by these previously tolerized T cells (Fig. 5, C and D). The net effect of anti-CTLA-4 administration was a notable 6% increase in the number of cells entering the cell cycle. Significantly, anti-CTLA-4 injection into the site of inflammation locally restored DTH effector function of previously tolerized T cells well after Ag priming without altering tolerance systemically (Fig. 6C). Interestingly, the administration of anti-CTLA-4 must coincide with antigenic stimulation, either at the time of immunization, at the time of DTH elicitation, or, as we previously demonstrated, during autoimmune inflammation (7). Once tolerized T cells have encountered Ag in the presence of anti-CTLA-4, they retain the ability to respond to subsequent Ag encounters (7).

It could be argued that the ability of anti-CTLA-4 treatment to restore immune function in vivo is not due to a direct effect on tolerant T cells, but due to its ability to enhance the proliferation and effector function of a small number of T cells that may have escaped tolerance induction. This explanation can be applied to every case in which anti-CTLA-4 has been shown to regulate

**FIGURE 6.** Regulation of tolerized T cell effector function by CTLA-4. A and B, DO11.10 TCR transgenic T cells (5 × 10⁶) were isolated and transferred into BALB/c mice. Twenty-four hours later, the recipient mice were injected with Sham-SP (A) or OVA323-SP (B) on day −5. On day 0, the mice were immunized with OVA323–339/CFA. On day +5, mice were ear challenged by the injection of 10 μg of OVA323–339. Cellular accumulation at the site of the peptide challenge was determined at 24 h by immunohistochemistry. Four ears per group were removed, embedded, frozen, and sectioned, and immunohistochemical identification of the KJ1-26⁺ component of the cellular infiltrate of the ear was undertaken. Positive staining is identified by the red fluorescence in conjunction with nuclear localization with 4',6'-diamidino-2-phenylindole (blue). C, Naive DO11.10 transgenic mice were treated with Sham-SP, OVA323-SP, or OVA323-SP + LPS on day −5. On day −2, CD4⁺ T cells were isolated from the spleens of the treated transgenic mice, and 5 × 10⁶ cells were transferred into naive BALB/c recipients. The BALB/c mice were immunized with OVA323–339/CFA on day 0. On day +5, OVA323–339-specific DTH responses were determined by 24-h ear-swelling assay with the modification that the OVA323–339 peptide was injected with 2 μg of either hamster control Ig or anti-CTLA-4 into the left and right ears, respectively. Results shown are the mean ear swelling of three mice per group ± SE and are representative of three separate experiments. *, Indicates DTH response significantly below that of Sham-SP-tolerized control, p < 0.01. Number above bar indicates percentage of tolerance.
tolerance induction (7, 8, 10). Although direct examination of the role of CTLA-4 on individual tolerized T cells using a tolerance-specific surface marker is not currently possible, the present results provide multiple lines of evidence indicating that anti-CTLA-4 has important direct effects on tolerized T cells. First, there are very few T cells that escape tolerance. It is possible to estimate the frequency of T cells that encountered Ag following treatment with Ag-SP. In Fig. 1, Ag-SP injection induced the activation of a large percentage (>95%) of Ag-specific T cells as determined by CD25 and CD69 expression. Thus, the vast majority of the transgenic T cells analyzed in these experiments have encountered tolerogen and should be considered tolerant. Correspondingly, the proportion of cells escaping tolerance must be extremely small. Second, the phenotype of the tolerized cells in response to subsequent immunization is that they have a reduced proliferative capacity as compared with nontolerized control (either naive (sham) or immunized) cells (Figs. 1A and 3). Proliferation beyond the second round of division was not seen as would be expected if the cells were naive or had otherwise escaped tolerance. Third, treatment with anti-CTLA-4 increased the percentage of cells entering the cell cycle (from 55% in control-treated groups vs 75% in the presence of anti-CTLA-4) (Fig. 5). Thus, anti-CTLA-4 promotes Ag-specific proliferation of previously tolerantized T cells in vivo. Finally, localized treatment with anti-CTLA-4 significantly and disproportionately increased effector DTH responses in tolerized mice in the anti-CTLA-4-treated ear, but not in the control-treated ear compared with nontolerized control mice (Fig. 6).

Collectively, these experiments demonstrate that CTLA-4 regulates tolerance maintenance in vivo both at the level of T cell expansion and T cell effector function, and that CTLA-4 appears to be required to actively maintain the unresponsive state. We propose that similar to memory T cells, tolerant T cells expressing CTLA-4 remain poised for an encounter with nominal Ag during ongoing inflammation, such as that seen in both immunization and autoimmune inflammatory settings. If a previously tolerized T cell (expressing CTLA-4) were to encounter Ag in the context of B7 (as in immunization or autoimmune inflammation), the presence of CTLA-4 would effectively raise the amount or quality of TCR engagement required for activation, thereby limiting T cell activity. In such a scenario, anti-CTLA-4 administration would restore the threshold of activation to normal levels.

References