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Acquired Thymic Tolerance: Role of CTLA4 in the Initiation and Maintenance of Tolerance in a Clinically Relevant Autoimmune Disease Model

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Injection of Ag into the thymus of adult animals induces specific systemic tolerance. The mechanisms of acquired thymic tolerance include anergy and the deletion of Ag-specific T cells. Here, we report that anergy to nominal Ag induced via acquired thymic tolerance requires CTL-associated Ag 4 (CTLA4) engagement. The role of CTLA4 in the induction and maintenance of tolerance was then investigated in the murine experimental autoimmune encephalomyelitis model. CTLA4 blockade abrogated the induction but not the maintenance phase of acquired thymic tolerance induced by intrathymic injection of myelin Ags. In addition, CTLA4 blockade had a restricted window of action prior to priming with Ag, which is consistent with the expression patterns of CTLA4 in vivo. We conclude that: 1) the induction of acquired thymic tolerance requires signaling through CTLA4 and 2) CTLA4 does not appear to be required for the maintenance of acquired thymic tolerance. This is the first report documenting the role of a CTLA4 negative signaling pathway in the induction of tolerance in an autoimmune disease model. The Journal of Immunology, 1999, 162: 761–765.

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3 Abbreviations used in this paper: MBP, myelin basic protein; CTLA4, CTL-associated Ag 4; i.t., intrathymic(ally); EAE, experimental autoimmune encephalomyelitis; HEL, hen egg lysozyme; LN, lymph node; MT, Mycobacterium tuberculosis.
were removed aseptically and mashed carefully to prepare single-cell suspensions. The cells from each group of mice were pooled for the proliferation and cytokine production assays as described previously (10, 11).

**EAE induction**

(SJL × PL/J)F₁ mice were immunized s.c. in the flanks with 1 mg/ml of mouse MBP peptide Ac₁–₁₁ emulsified in an equal amount of CFA containing 2 mg/ml CFA (Difco) (total amount of Ac₁–₁₁ injected is 100 μg/mouse). The animals received 200 ng of pertussis toxin i.p. (List Biological Laboratories, Campbell, CA) at 24 h postimmunization. Scoring of clinical disease was performed daily as described previously (21). In this model, animals develop acute disease by day 8–12 postimmunization. In our study, the disease incidence is 100%, the day of onset is day 10.78 ± 0.3, and the mean maximal grade is 3.0 ± 0.3 (mean ± SEM from 28 mice in several experiments). The acute disease is followed by a clinical remission, and the mice subsequently have one or more relapses.

**Ab treatment**

Each mouse received an i.p. injection of 100 μg of hamster anti-mouse CTLA4 Ab from ascites of B cell hybridoma (clone 4F10, kindly provided by Dr. Jeffrey Bluestone, University of Chicago, Chicago, IL (14)); the control group received equal amount of hamster Ig i.p. Ab was administered in single or multiple doses as outlined in Results.

**Cell cultures**

Cell suspensions were washed twice before resuspension in DMEM (Bio-Whittaker) and supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate (all from BioWhittaker), penicillin (100 U/ml), streptomycin (100 μg/ml), and 20 μM 2-ME (Sigma, St.Louis, MO). For splenocytes, RBCs were lysed with ACK lysing buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA). Cells (4 × 10⁶ cells/200 μl/well) were cultured in round-bottom microtiter plates (Costar, Cambridge, MA) and stimulated with OVA or Mycobacterium tuberculosis (MT); unstimulated wells served as background response. Proliferation was measured using a standard 72-h lymphocyte proliferation assay. For cytokine production, cell-free supernatants were collected after 48 h for the measurement of IL-2, IFN-γ, IL-4, and IL-10 production. The results of the proliferative and cytokine studies were similar for both splenocytes as well as lymphocytes from draining LN cells; therefore, only data for LN cells are shown.

**ELISA of cytokines**

A quantitative ELISA for IL-2, IFN-γ, IL-4, and IL-10 was performed using paired mAbs specific for the corresponding cytokine according to the manufacturer’s recommendations (PharMingen, San Diego, CA). Standard curves were generated using known amounts of purified murine rIL-2, rIL-4, rIFN-γ, or rIL-10 (PharMingen).

**Statistical analysis**

For a comparison of proliferative responses and cytokine production between experimental groups across three to four experiments, we used non-parametric Kruskal-Wallis and Mann-Whitney tests. For a comparison of clinical disease, we used the Mann-Whitney test to compare grade and onset and Fisher’s exact test for incidence rates.

**Results**

**Acquired thymic tolerance to nominal Ag and autoantigen**

An injection of OVA into the thymus of adult BALB/c mice induces Ag-specific tolerance. As seen in Fig. 1, LN cells from animals injected i.t. with OVA before immunization have decreased proliferation and IL-2 and IFN-γ production after stimulation with OVA in vitro compared with LNs from animals injected with HEL i.t., as described previously (10). MT was used in vitro as a control Ag because the mice were immunized with CFA but not tolerized to MT. Thus, the proliferation and cytokine production by lymphocytes to MT provide evidence of the Ag specificity of tolerance. Similarly, an injection of HEL into the thymus of OVA-immunized mice shows the specificity of the tolerizing Ag (9, 10). For an in vivo model, we used (SJL × PL/J)F₁ mice immunized with Ac₁–₁₁/CFA. In this model, an i.t. injection of Ac₁–₁₁ peptide but not PBS induces protection from clinical EAE (Fig. 2). The incidence of disease was six of six for PBS mice and three of four for i.t.-tolerized mice. The mean score was 3.3 ± 0.4 for the PBS group and 1.1 ± 0.6 for the i.t.-tolerized group. Furthermore, this treatment prevents relapsing disease. Interestingly, mice immunized with MBP/CFA and tolerized by an i.t. injection of Ac₁–₁₁ peptide are protected from acute disease but not from relapses, suggesting that acquired thymic tolerance is epitope-specific (data not shown) as we have reported in the Lewis rat model.

**Role of CTLA4 signaling in acquired thymic tolerance**

To investigate the role of the CTLA4 molecule in acquired thymic tolerance, we used a blocking anti-CTLA4 Ab to inhibit signal transduction through the CTLA4–B7 pathway. Studies with this Ab (clone 4F10) and with its Fab fragments have been reported and have shown no functional difference in CTLA4 blockade in vitro or in vivo between the Ab and its Fab fragment (22–24). Anti-CTLA4 was administered to BALB/c mice at different timepoints.
The mean score was 3.3 ± 0.6 for the PBS group and 3 of 4 for the i.t.-tolerized group. The mean score was 3.3 ± 0.4 for the PBS mice and 1.1 ± 0.6 for the i.t.-tolerized mice. Data from this experiment are included in Table 1. This experiment was repeated five times with similar results. The $x$-axis represents the days of observation, and the $y$-axis represents the mean clinical score of each group.

FIGURE 2. Effects of an i.t. injection of Ac1–11 peptide of MBP on the course of EAE. (SJL × PLJ)F1 mice received 100 μg of Ac1–11 (O) or PBS (■) i.t. 48 h before immunization with Ac1–11/CFA. There were six mice in the PBS group and four mice in the i.t.-tolerized group. Incidence of disease was six of six for the PBS group and three of four for the i.t.-tolerized group. The mean score was 3.3 ± 0.4 for the PBS mice and 1.1 ± 0.6 for the i.t.-tolerized mice. Data from this experiment are included in Table 1. This experiment was repeated five times with similar results. The $x$-axis represents the days of observation, and the $y$-axis represents the mean clinical score of each group.

FIGURE 3. Effects of blocking CTLA4 signaling on the induction of acquired thymic tolerance. BALB/c mice were i.t. injected with OVA, except for both the control group, which was not injected i.t., and the group that received HEL i.t. All groups were immunized with OVA/CFA after 48 h. Anti-CTLA4 Ab was administered at a dose of 100 μg per mouse on the days noted on the $x$-axis. Control Ig was administered on days –3, –2, –1, 0, 3, and 6 relative to immunization. The group injected with HEL i.t. received anti-CTLA4 on days –3, –2, and –1. Draining LN cells were obtained on day 10 postimmunization. Cells were pooled from three mice per group and stimulated with OVA at 25 μg/ml; proliferation, IFN-γ production, and IL-2 production were measured. Fig. 4 shows the results of stimulation with MT at 25 μg/ml. The data clearly show that anti-CTLA4 administration on day 3 did not enhance proliferation or IFN-γ or IL-2 production compared with controls.

Role of CTLA4 signaling in the induction and maintenance of acquired thymic tolerance in EAE

To investigate the role of CTLA4 signaling in the induction and maintenance of acquired thymic tolerance in a clinically relevant autoimmune disease model, we used the relapsing EAE model. Based on the data obtained in the OVA system, we investigated the effect of anti-CTLA4 administration on day 3 postimmunization (tolerance induction phase) and on later timepoints to evaluate the maintenance phase of tolerance. As shown in Table I, administering anti-CTLA4 to i.t. tolerized mice on day 3 postimmunization reversed tolerance, whereas administering anti-CTLA4 on days 10, 12, and 14 postimmunization did not reverse protection (Fig. 5 and Table I). The administration of anti-CTLA4 blocking Ab was reported to worsen EAE (24, 25). However, in our model the reversal of protection seen on day 3 postimmunization cannot be attributed to a worsening of disease. At the doses and protocols used, we observed a slight shortening of disease latency and slightly increased mortality (not statistically significant). The same degree of worsening was observed when anti-CTLA4 was administered on day 3 or on days 10, 12, and 14 postimmunization in the non-tolerized animals, but reversal of tolerance was only observed in the day 3 anti-CTLA4-treated thymically injected animals. These data and the observations by Perez et al. (18) in a model of peripheral tolerance to nominal Ag and by Walunas et al. (26) in a model of tolerance to superantigen indicate that CTLA4 blockade is abrogating the induction of tolerance rather than just worsening the immune response.

These results suggest that once the T cell is rendered unresponsive, it is resistant to the effects of CTLA4 blockade. Thus, our data show that CTLA4 negative signaling is not necessary for the maintenance phase of tolerance and are consistent with the findings of Perez et al. for peripheral tolerance to nominal Ag (18).
Discussion

The induction of specific tolerance with an i.t. injection of Ag was initially described by Waksman and colleagues in the 1960s–1970s (27–29) and recently applied to several transplantation (8, 30) and autoimmune models (1–5). In the autoimmune disease model EAE, an i.t. injection of MBP or its major encephalitogenic peptide (p71–90) but not a nonencephalitogenic peptide (p21–40) of MBP prevents disease in the Lewis rat (4); this finding is associated with an Ag-specific suppression of proliferation of the LN T cells of these animals (4). We have shown previously that there is a down-regulation of Th1 cytokines in the brain without evidence of Th2 up-regulation (4). We have also shown that thymic dendritic-cell-enriched cells mediate the induction of acquired thymic tolerance (9), and that the protective effect of thymic dendritic cells is abrogated in thymectomized animals, suggesting that the interaction of dendritic cells and T cells occurs in the thymus (9). More recently, we reported that an i.t. injection of Ag in a mouse model induces Th1 cell unresponsiveness and prevents the peripheral expansion of Ag-specific CD4+ T cells in vivo (10). Injection of OVA into the thymus of OVA TCR-transgenic mice induces apoptosis of immature thymocytes and anergy of more mature thymocytes (11). Thus, our data support the hypothesis that activated cells circulate to the thymus, where they are inactivated by anergy and/or deletion (10, 11). CTLA4 negative signaling reportedly plays a role in peripheral tolerance to nominal Ags (18, 26). In this report, we investigated the role of CTLA4 signaling in acquired thymic tolerance.

Our results clearly show that CTLA4 signaling is required during the induction of but not the maintenance of acquired thymic tolerance. Administration of anti-CTLA4 on day 3 postimmunization reverses acquired thymic tolerance. One could argue that blocking CTLA4 signaling enhances immunity rather than abrogates tolerance. Several observations in our study counter this hypothesis. First, our in vivo protocol of anti-CTLA4 administration to control (nontolerized) animals did not worsen disease. The differential effect of anti-CTLA4 administration on day 3 vs days 10–14 is another argument against this hypothesis. Second, we demonstrate in our in vitro experiments that the administration of anti-CTLA4 on day 3 postimmunization did not augment the immune response to MT (an Ag to which the mice were not tolerized). Thus, there is no evidence that administration of anti-CTLA4 on day 3 postimmunization augments the immune response or worsens disease. Our data demonstrate that anti-CTLA4 administered on day 3 postimmunization prevents the anergy normally induced by acquired thymic tolerance.

We found that there is a narrow window for CTLA4 signaling in acquired thymic tolerance. Our in vitro data suggest that this window is between days 3 and 6 postimmunization. Interestingly, administration of anti-CTLA4 around the day of immunization did not abrogate the induction of tolerance. One would have expected that an IgG Ab might persist in the circulation so that some effect can be seen on day 3. However, several factors determine the efficacy of Ab blockade in vivo, including τ/2 (shorter with a xenogenic Ab), peak serum concentration, tissue distribution, and the level of expression of the target molecule. In our experiments with nominal Ag, the administration of anti-CTLA4 on day 0 may not provide an optimal tissue level at the time of peak CTLA4 expression. This is supported by data from Bluestone (20) showing the delayed expression of CTLA4 after Ag priming in vivo. Interestingly, a similar narrow window for anti-CTLA4 action has been reported in a model of autoimmune diabetes in which the administration of anti-CTLA4 during a critical period before the onset of insulitis leads to a more rapid onset of diabetes (31).
Our findings are different from those reported by Perez et al. (18) in a model of peripheral tolerance in vivo in which the administration of anti-CTLA4 at the time of Ag administration before priming abrogated the induction of tolerance. Alternatively, our observations are in keeping with our hypothesis of the mechanisms of acquired thymic tolerance: peripheral activation of Ag-specific T cells is followed by a migration of activated T cells to the thymus (10), where they interact with thymic dendritic cells and either become anergic or are deleted (11). Anergy induced via acquired thymic tolerance is not reversed by the addition of IL-2 in vitro (10), suggesting that this anergy is not a form of clonal ignorance but specific inactivation (18). This possibility is supported by the reversal of tolerance seen after blocking B7-CTLA4 interaction. Consequently, we hypothesize that blocking CTLA4 signaling can reverse acquired thymic tolerance only at the time at which the tolerizing signal is given to the T cell (i.e., after the initial activation through immunization). Therefore, the role of CTLA4 signaling in the induction of tolerance in different models is dependent upon the exact mechanisms mediating the tolerant state in vivo.

Our findings are the first to demonstrate an important role for CTLA4 negative signaling in the induction of tolerance in a clinically relevant autoimmune disease model in vivo. Our data have relevant clinical implications, because they suggest that the development of novel strategies in humans should take into account the importance of keeping T cell signaling through CTLA4 intact if true tolerance is to be achieved.

**References**


