Brief Definitive Report

CTLA-4: A Negative Regulator of Autoimmune Disease

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Summary

CTLA-4, a CD28 homologue expressed on activated T cells, binds with high affinity to the CD28 ligands, B7-1 (CD80) and B7-2 (CD86). This study was designed to examine the role of CTLA-4 in regulating autoimmune disease. Murine relapsing-remitting experimental autoimmune encephalomyelitis (R-EAE) is a demyelinating disease mediated by PLP139-151-specific $CD4^+$ T cells in SJL/J mice. Anti-CTLA-4 mAbs (or their F(ab) fragments) enhanced in vitro proliferation and pro-inflammatory cytokine production by PLP139-151-primed lymph node cells. Addition of either reagent to in vitro activation cultures potentiated the ability of T cells to adoptively transfer disease to naive recipients. In vivo administration of anti-CTLA-4 mAb to recipients of PLP139-151-specific T cells resulted in accelerated and exacerbated disease. Finally, anti-CTLA-4 treatment of mice during disease remission resulted in the exacerbation of relapses. Collectively, these results suggest that CTLA-4 mediates the downregulation of ongoing immune responses and plays a major role in regulating autoimmunity.

T cell activation requires the delivery of at least two signals to the T cell by the antigen-presenting cell (APC). One signal is antigen-specific and is provided by the ligation of the T cell receptor (TCR) by antigen-MHC complexes, whereas the second "costimulatory" signal may be provided by the B7 family of molecules, B7-1 (CD80) or B7-2 (CD86), expressed on the APC. Interactions between CD28 on the T cell and the B7 molecules on the APC are critical, both in vivo and in vitro, in preventing T cell unresponsiveness (1–4). The discovery of the CD28 homologue, cytotoxic T lymphocyte associated molecule-4 (CTLA-4), by subtractive mRNA cloning of cytotoxic T cells (5) has increased the complexity of costimulatory interactions. CTLA-4 binds with higher affinity to the B7-1 and B7-2 molecules (6). CTLA-4Ig, a soluble recombinant form of CTLA-4, is a potent inhibitor of B7/CD28 interactions and inhibits in vivo antigen-specific responses (2, 3, 7, 8). However, the regulatory function of the native CTLA-4 molecule on the T cell surface has only recently been appreciated.

The cytoplasmic tail of human and murine CTLA-4 is highly conserved (9), suggesting that it is involved in an important signaling function. Surface expression of CTLA-4 seems to be a tightly regulated event with transient expression on activated T cells, peaking at 48–72 h after activation (10, 11). Initial studies suggested that anti-CTLA-4 antibodies could synergize with anti-CD3 and anti-CD28 to enhance T cell proliferation (10, 12). Thus, it was concluded that CTLA-4 was an additional costimulatory molecule that had functions cooperative with those of CD28. However, recent studies have indicated that CTLA-4 may, in fact, serve as a negative regulator of T cell activation. We have shown that soluble anti-CTLA-4 mAb as well as its F(ab) fragments increased T cell proliferation in murine MLR responses (11). Additionally, intact cross-linked anti-CTLA-4 mAb, but not its F(ab) fragments, could inhibit proliferation in T cells stimulated with anti-CD3 and anti-CD28 (11, 13). Thus, it is likely that CTLA-4 functions to downregulate T cell responses such that blockade of CTLA-4/B7 interaction results in enhanced T cell activation. Other studies also support a negative regulatory role for CTLA-4. Cross-linking of CTLA-4 after the ligation of CD3 and CD28 strongly inhibited proliferation and IL-2 production (14). In vivo treatment of mice with anti-CTLA-4 was shown to enhance clonal expansion of antigen-specific T cells after immunization (15) and enhance anti-tumor immunity (16). In fact, CTLA-4 knockout mice express a lymphoproliferative disorder resulting in massive infiltration of several organs leading to early death (17, 18). Together, these studies support an essential role for CTLA-4 in the downregulation of potentially autoaggressive T cells.

The present study was conducted to directly examine the role of CTLA-4 in regulating autoantigen-specific responses in autoimmune disease. Relapsing-remitting experimental

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autoimmune encephalomyelitis (R-EAE) is a well-established murine model for multiple sclerosis (MS) characterized by $CD4^+$ T cell–mediated demyelination in the central nervous system (CNS) (19). R-EAE is induced in SJL/J mice by immunization with the immunodominant epitope on proteolipid protein (PLP139-151) or by the adoptive transfer of PLP139-151-specific T cells (20). The clinical disease is characterized by an acute paralytic phase followed by recovery and subsequent relapses accompanied by development of T cell responses to endogenous epitopes on myelin proteins (e.g., PLP178-191), a phenomenon termed epitope spreading (21, 22). CD28/B7-mediated costimulatory interactions play a major role in the establishment and progression of R-EAE. Treatment of mice with CTLA-4Ig has been shown to inhibit disease induction (8). Anti-B7-1 or anti-B7-2 treatments have differential effects on autoimmune diseases including R-EAE (23, 24) and autoimmune diabetes (25). Together, these studies emphasize the complexity of costimulatory interactions between B7 molecules and CD28 or CTLA-4. In this study, we have directly addressed the role of CTLA-4 in modulating PLP139-151-specific T cell responses and in regulating disease using an anti-CTLA-4 mAb, known to prevent CTLA-4/B7 interactions. Both intact Ig and F(ab) fragments had similar effects in all the responses studied, suggesting that these reagents were blocking CTLA-4-mediated events. First, anti-CTLA-4 mAb enhanced in vitro proliferation and production of pro-inflammatory cytokines by PLP139-151-primed lymph node cells. Second, addition of anti-CTLA-4 to in vitro activation cultures resulted in potentiation of the ability of these cells to adoptively transfer R-EAE to naive recipients. Third, in vivo blockade of CTLA-4 in recipient mice, after adoptive transfer of primed cells, also resulted in exacerbation of disease. Finally, treatment of mice during remission after the acute phase of R-EAE resulted in greater incidence and severity of relapses. These results indicate that CTLA-4 has an important role in the downregulation of T cell responses in autoimmune disease and may be a major regulatory player in the control of ongoing immunopathology.

Materials and Methods

Mice. Female SJL/J mice, 6–7 wk old, were purchased from Harlan Laboratories (Indianapolis, IN), housed in the Northwestern animal care facility, and maintained on standard laboratory food and water ad libitum. Paralyzed mice were afforded easier access to food and water.

Peptides. PLP139-151 (HSLGKWLGHPDKF) was purchased from Peptides International (Louisville, KY). Amino acid composition was verified by mass spectrometry and purity (>98%) was assessed by HPLC.

In Vitro Proliferation and Cytokine Assays. Mice were immunized subcutaneously with 100 μl of a Complete Freund's Adjuvant (CFA) emulsion containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Labs. Inc. Detroit, MI) and 25 μg of PLP139-151 distributed over three spots on the flank. On day 10, draining lymph node cells were harvested and cultured in 96-well microtiter plates at a density of 5×10^5 cells/well in a total volume of 200 μl Dulbecco's Modified Eagle Medium (DME) containing 7% fetal bovine serum, 1 mM glutamine, 1% Pen-Strep, 1 mM nonessential amino acids and 5×10^{-5} M 2-mercaptoethanol (complete DME-7; all products from Sigma Chem. Co., St. Louis, MO). Cells were allowed to incubate at 37°C for 60 min with the indicated antibodies (10 μg/ml) before the indicated doses of antigen were added. Cultures were pulsed with 1 μCi of [^{3}H]TdR after 72 h, harvested at 96 h and [^{3}H]TdR uptake determined by scintillation counts. Results are expressed as mean of triplicate cultures ± SEM (background counts subtracted). Supernatants collected at 24 and 48 hours from replicate cultures were assayed for IL-2, IL-4, and IFN-γ levels using ELISA Mini-Kits (Endogen, Cambridge, MA). Minimal detection limits of the assays were ~10 pg/ml for IL-2 and IL-4 and 30 pg/ml for IFN-γ.

Flow Cytometry. FACS® analysis was performed using the FACScan® flow cytometer as described previously (11, 24). PE-conjugated anti-CD3 and FITC-conjugated control Ig were purchased from PharMingen (La Jolla, CA).

Induction of R-EAE by Active Immunization with PLP139-151. Mice were immunized with 80 μg PLP139-151 in CFA as previously described (20).

Adoptive Transfer of R-EAE. Donor mice were immunized with 25 μg of PLP139-151 in CFA. Draining lymph node cells were harvested on day 10 and cultured in either T25 (total volume: 15 ml) or T75 (30 ml) flasks at 8×10^6 cells/ml with 20 μg/ml of PLP139-151. In antibody-containing cultures, cells were incubated at 37°C for 60 min with the indicated mAbs (10 μg/ml) before the addition of antigen. On day 4, cells were washed in PBS, counted, and transferred i.p. into naive recipients in a total volume of 0.5 ml PBS. Recipient mice received a suboptimal dose of $6–7 \times 10^6$ live cells each.

Antibody Treatments. The following mAbs were employed: hamster control Ig (Cappel Research Products, Cochranville, PA) and the anti-CTLA-4 mAb (UC11-4F10) (11). Antibodies and F(ab) fragments were produced and purified as previously described (11, 25). Mice were treated, as indicated, either starting on the day of adoptive transfer or after recovery from the initial paralytic episode of R-EAE (~25 d post-immunization). 50 μg of antibody was administered i.p. every other day for five treatments (250 μg total) and mice were monitored for clinical signs of disease for an additional 20–40 d.

Clinical Evaluation. Clinical severity was assessed on a 0 to 5 scale as described (24). The data are plotted as the daily mean clinical score for all animals in a particular treatment group. A relapse was defined as a sustained increase of at least one full grade in clinical score after the animal had previously improved at least a full clinical score and had stabilized. Mean maximal severity is the mean of the highest clinical score reached by mice within an experimental group which displayed clinical signs.

Statistical Analyses. Comparison of the percentage of animals showing clinical disease and/or relapses between any two groups of mice were analyzed by χ^2 using Fisher's exact probability. Comparisons of the mean day of onset of disease/relapse, and mean peak disease severity between any two groups of mice were analyzed by the Student's t test. P values <0.05 were considered significant.

Results and Discussion

Anti-CTLA-4 mAb Enhances PLP139-151-Specific In Vitro Proliferative and Cytokine Responses. Recent in vitro studies have employed allogeneic MLR or anti-CD3 stimulation to assess the expression and function of CTLA-4 on T

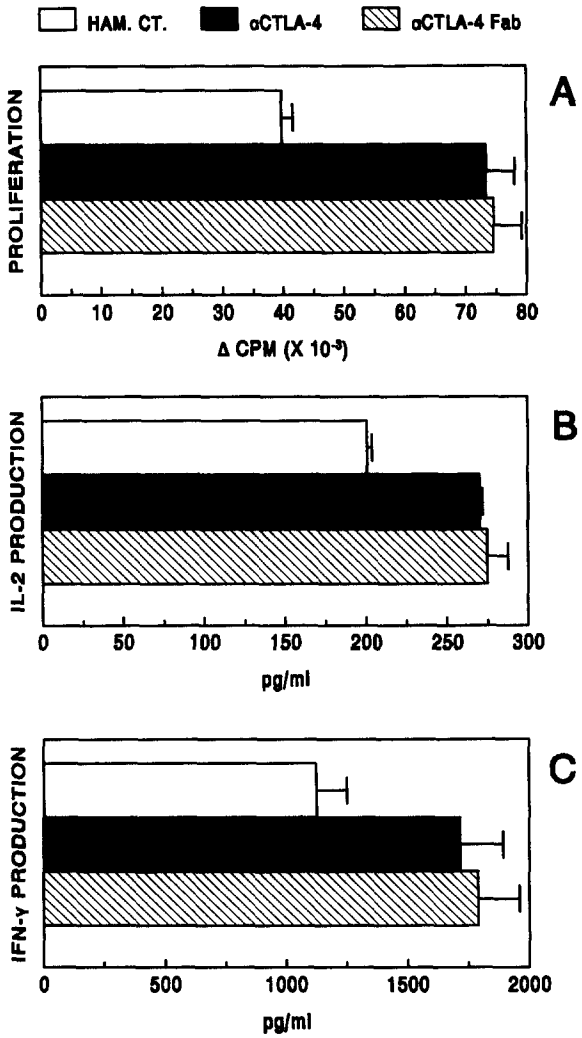

Figure 1. Anti-CTLA-4 mAb enhances in vitro proliferation and cytokine production by PLP139-151-primed lymph node cells. (*A*) Draining lymph node cells from PLP139-151/CFA-primed mice were cultured with 0.5 μM PLP139-151 and 10 μg/ml of control or anti-CTLA-4 antibodies. Data represents the mean thymidine uptake of triplicate cultures and is plotted as Δ counts per minute ± SEM (background counts [1712 ± 138] subtracted). These results are representative of six separate experiments. (*B* and *C*) Supernatants from replicate experiments were collected after 24 h of culture and assayed for IL-2 and IFN-γ levels by ELISA. Data is plotted as mean cytokine levels in pg/ml ± SD at a peptide dose of 0.5 μM (IL-2) or 15 μM (IFN-γ). Results are representative of three separate experiments.

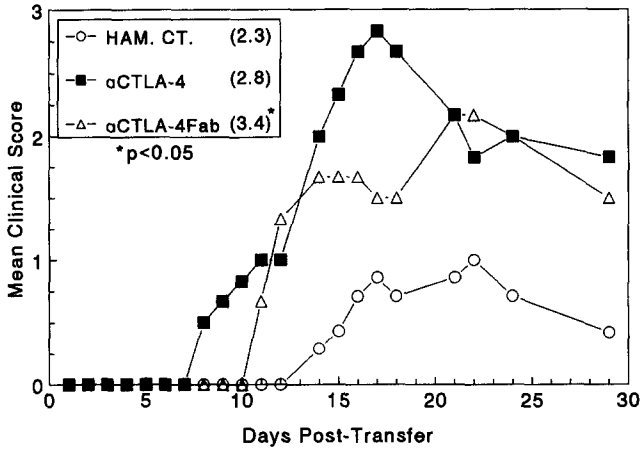

Figure 2. Addition of anti-CTLA-4 mAb to in vitro activation cultures enhances the ability to transfer R-EAE. Control or anti-CTLA-4 antibodies were added to in vitro activation cultures of PLP139-151-primed cells. On day 4, 6×10^6 total live cells were transferred i.p. into naive recipients. Mice were then graded for clinical signs of R-EAE. Data is plotted as mean clinical score vs. days post-transfer. Disease incidences in the different groups were 4/7(Control), 6/6 (anti-CTLA-4) and 5/6 (anti-CTLA-4 F(ab)). Mean days of onset were 17.8, 12.5*, and 14.2* d respectively (*$P < 0.05$). Figures in parentheses indicate mean maximal clinical severity in each group (*$P < 0.05$). Results are representative of four separate experiments.

cells (10, 11, 14). To address the functional role of CTLA-4 in nominal antigen-specific immune responses, mice were immunized with the immunodominant PLP139-151 epitope and the effects of anti-CTLA-4 mAb (10 μg/ml) on in vitro proliferation and cytokine production by primed lymph node cells were determined. As seen in Fig. 1 *A*, the anti-CTLA-4 mAb significantly enhanced PLP139-151-specific proliferation. Higher concentrations of antibodies enhanced proliferative responses even further (data not shown). 24-h supernatants from replicate cultures were assayed for IL-2, IL-4, and IFN-γ levels by ELISA. Anti-CTLA-4 mAb resulted in significantly enhanced IL-2 and IFN-γ production (Fig. 1, *B* and *C*). No IL-4 was detected in any of the supernatants (data not shown). Similar patterns of enhanced proliferation and cytokine production were seen with varying peptide doses and after varying culture times. The F(ab) fragments and the intact anti-CTLA-4 antibody had the same enhancing effect, consistent with previous studies (11, 15), suggesting that the enhancement of proliferation and cytokine production was due to the blockade of CTLA-4-mediated downregulatory events.

Addition of Anti-CTLA-4 mAb to In Vitro Activation Cultures Enhances the Ability of PLP139-151-specific T Cells to Transfer R-EAE to Naive Recipients. We next tested whether blockade of CTLA-4/B7 ligation altered the generation of encephalitogenic PLP139-151-specific cells. PLP139-151-primed lymph node cells were activated in vitro in the presence of control Ig or anti-CTLA-4 antibodies. Encephalitogenicity of the resulting cells was then assessed by the expression of clinical disease after the adoptive transfer of a suboptimal dose of 6×10^6 viable cells to naive recipients. Cells cultured with either anti-CTLA-4 or its F(ab) fragments were significantly more efficient at transferring R-EAE (Fig. 2). Mice receiving anti-CTLA-4-treated cells demonstrated an accelerated disease course (mean day of onset: 12.5* d and 14.2* d in antibody-treated groups vs. 17.8 d in controls; *$P < 0.05$) and a higher mean maximal disease severity (2.3 mean peak score in controls vs. 2.8 in the anti-CTLA-4-treated group and 3.4* in the F(ab)-treated group; *$P < 0.05$). These results indicate that the blockade of CTLA-4/B7 interactions in the activation cultures resulted in an enhanced encephalitogenicity of the

cells transferred to naive recipients. Since anti-CTLA-4 mAb treatment also resulted in elevated proliferation and cytokine production by PLP139-151-specific T cells (Fig. 1), the enhanced disease course may be due to the transfer of a higher number of effector T cells and/or of effector cells capable of producing enhanced levels of pro-inflammatory cytokines.

In Vivo Treatment with Anti-CTLA-4 mAb Exacerbates Adoptive R-EAE. We next examined whether CTLA-4-mediated events downregulated PLP139-151-specific T cells in vivo. Activated PLP139-151-specific cells were adoptively transferred to naive SJL/J recipients. Recipient mice were then treated with control Ig, intact anti-CTLA-4, or anti-CTLA-4 F(ab) fragments. Anti-CTLA-4 mAb treatment exacerbated clinical disease (Fig. 3). Disease onset was accelerated (mean day of onset (MDO): 7.0* d in anti-CTLA-4 treated group vs. 13.0 d in controls; $^*P < 0.01$) and the mean maximal severity of the anti-CTLA-4-treated groups was significantly greater (3.0 in controls vs. 4.6* in anti-CTLA-4-treated groups; $^*P < 0.01$). In vivo treatment with anti-CTLA-4 F(ab) fragments similarly accelerated and exacerbated disease with a MDO of 10.0* d and a mean maximal severity of 3.7*; $^*P < 0.05$ (data not shown). Thus, it appears that CTLA-4-mediated events play a role in the downregulation of the autoimmune effector functions and/or the in vivo expansion of the adoptively transferred cells. Flow cytometric analyses of PLP139-151-primed lymph node cells activated in culture showed upregulation of surface CTLA-4 expression on T cells (data not shown). Lymph node cells taken ex vivo from primed mice showed little CTLA-4 expression (time 0 h) which peaked after in vitro activation at about 72 h of culture, as described before in other systems (11). At the time of adoptive transfer (96 h), a small, but significant population (~10%) of T cells, showed surface CTLA-4 expression. Treatment of the recipient animals starting on the day of transfer could result in the enhancement of pro-inflammatory phenomena due to blockade of CTLA-4-mediated downregulatory events in this population of effector cells, or on other T cells within the transfer population which may upregulate CTLA-4 after presentation of self peptides in the CNS.

Anti-CTLA-4 mAb Treatment Exacerbates Relapses in R-EAE. R-EAE induced by immunization with PLP139-151 exhibits a relapsing and remitting disease course. The acute phase of disease is due to the activity of $CD4^+$ T cells specific for the PLP139-151 epitope. However, we have recently shown (21) that the primary disease relapse is principally due to the activity of T cells specific for an endogenous, non-cross-reactive epitope on PLP (PLP178-191) which are activated as a result of recognition of myelin epitopes released during acute disease, a phenomenon known as epitope spreading. Tolerization of PLP178-191-specific T cells (unpublished observations) or inhibition of PLP178-191-specific responses by the blockade of B7-1-mediated costimulatory signals during disease remission blocks disease relapses (24). To assess the role of CTLA-4 in disease relapses, mice were treated with anti-CTLA-4 mAb during remission, i.e., after they had recovered from the acute phase of active disease. Anti-CTLA-4 treatment resulted in an increased disease severity and a higher incidence of relapses (Fig. 4). Four out of the seven mice in the control

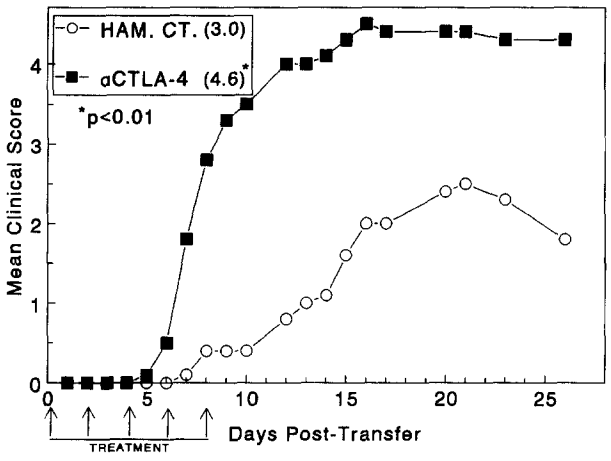

Figure 3. In vivo CTLA-4 blockade exacerbates adoptive R-EAE. R-EAE was initiated by adoptive transfer of PLP139-151-specific T cells. Starting on the day of transfer, mice were treated i.p. with either control or anti-CTLA-4 mAbs and graded for clinical signs of EAE. Data is plotted as the mean clinical score vs. days post-transfer. Disease incidences were 8/8 (control group) and 8/8 (anti-CTLA-4-treated group). Mean days of onset were 13.0 and 7.0* d, respectively ($^*P < 0.01$). Figures in parentheses indicate mean peak clinical severity in each group ($^*P < 0.01$). Results are representative of three separate experiments.

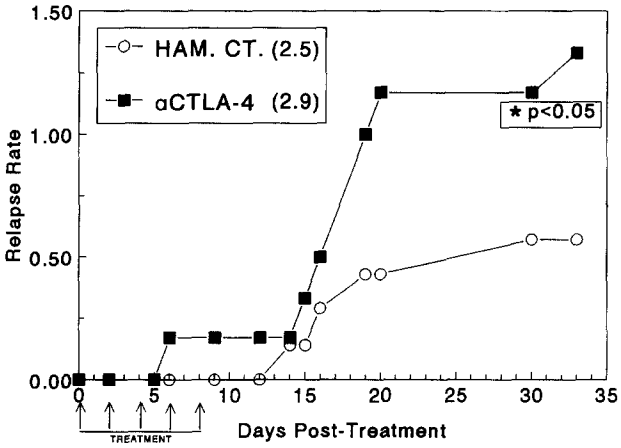

Figure 4. Anti-CTLA-4 treatment exacerbates disease relapses in R-EAE. Active R-EAE was induced by priming with PLP139-151/CFA. After recovery from the acute phase of disease (~28 d post immunization), mice were treated with either control Ig or intact anti-CTLA-4 mAb. Data is plotted as the relapse rate (total number of relapses in a group divided by the total number of mice in that group) vs. days post-treatment initiation. 4/7 mice in the control group showed relapses, whereas 6/6 mice in the anti-CTLA-4-treated group relapsed (two mice relapsed two times each making a total of 8 relapses in 6 mice). Figures in parentheses indicate the mean peak clinical score of the relapses. These results are representative of two separate experiments. *Number of relapses are significantly greater than that of the hamster Ig controls, $P < 0.05$.

group showed relapses whereas six out of six mice in the anti-CTLA-4 treated group relapsed, with two mice showing two relapses each ($P < 0.05$).

Collectively, the data presented in this paper support the hypothesis that CTLA-4 is a major negative regulator of autoimmune T cell function both in vitro and in vivo and emphasizes the complicated nature of costimulatory interactions. We have shown that the blockade of CTLA-4 interactions with its ligands resulted in the enhancement of every effector function studied, including proliferation, pro-inflammatory cytokine production, encephalitogenicity, and the ability to maintain ongoing autoimmune disease. These results are consistent with a model of costimulatory interactions, where initial activation of T cells after antigen exposure is mediated by CD28/B7 interactions and leads to the proliferation and differentiation of effector T cells. Activated T cells express CTLA-4 on their surface, which can bind the same B7-family ligands with higher affinity and result in the downregulation of the T cell response. The net T cell response would then be a result of a balance between CD28 and CTLA-4-mediated events.

Our recent results show that, during the course of R-EAE, there is preferential upregulation of B7-1, as compared to B7-2, both in the CNS and in the splenic lymphoid compartment (24). Such dynamic changes in costimulatory ligand expression during a chronic inflammatory autoimmune response may reflect an attempt at downregulating a destructive response. Based on the parallel late upregulation of B7-1 and CTLA-4, it has been suggested that B7-1 may be the preferred ligand for CTLA-4 (26). Our preliminary data suggest that there are dramatic differences in B7 ligand expression depending on the lymphoid tissue examined. It is tempting to speculate that certain compartments of the immune system may be involved in providing an environment which promotes pro-inflammatory events, whereas other compartments may be primarily responsible for the ultimate downregulation of T cell responses. Our current studies are directed towards addressing these issues by carefully following the expression and functions of B7 molecules and their T cell ligands in animals with an apparent self regulating autoimmune disease such as observed during relapsing-remitting EAE.

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