Kinetics of Expression of Costimulatory Molecules and Their Ligands in Murine Relapsing Experimental Autoimmune Encephalomyelitis In Vivo

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Kinetics of Expression of Costimulatory Molecules and Their Ligands in Murine Relapsing Experimental Autoimmune Encephalomyelitis In Vivo

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We studied the kinetics of expression of costimulatory molecules and cytokines in the central nervous system (CNS) in murine relapsing experimental autoimmune encephalomyelitis (EAE). During the natural course of EAE, B7-2 expression in the CNS correlated with clinical signs, while B7-1 was exclusively expressed during remissions. Interestingly, B7-1 was expressed on infiltrating mononuclear cells as well as neuronal cells in the CNS. In the periphery, B7-1 expression on APCs peaked with clinical disease but decreased on T cells. CD28 and CTLA4 molecules, the two known ligands for B7-1 and B7-2, had distinct expression patterns in the CNS; CD28 was highly expressed and correlated with B7-2 expression on APCs (macrophages/microglia as well as astrocytes) and with the clinical signs of EAE. CTLA4, on the other hand, was expressed by substantially fewer cells during the effector phase of disease and peaked during remission, which is consistent with the emerging role of this molecule in the termination of immune responses. The expression of CD40 and CD40L in the CNS was increased during clinical attacks. The expression of IL-12, IFN-γ, and TNF-α correlated with disease activity and severity, while TGF-β was the only factor that was up-regulated during the recovery phase. Interestingly, TGF-β was also expressed by neurons during remission. This is the first study demonstrating the kinetics of the in vivo expression of costimulatory molecules, their ligands, and cytokines in an autoimmune disease model characterized by remissions and relapses. Our data suggest that the targeting of costimulatory molecules to block an immune response must take into account the expression patterns in the target organ. The Journal of Immunology, 1998, 161: 1104–1112.

Signal transduction through the CD28-B71/B72 and the CD40 ligand (CD40L) CD40 costimulatory pathways plays an important role in the initiation of T cell responses (1–4); recent work indicates that these two pathways are closely related and cross-regulated (5, 6). In fact, blockade of either pathway alone has been shown to prevent autoimmune disease and transplant rejection in several experimental models (reviewed in Ref. 7). Additionally, there is evidence that blockade of both pathways may be synergistic. Indeed, the clinical development of re-agents to block either pathway is ongoing (7).

Experimental autoimmune encephalomyelitis (EAE) is a demyelinating central nervous system (CNS) disease that is initiated by encephalitogenic CD4 T cells (8, 9) which are specific to myelin Ags. Th2 cells are not encephalitogenic (10), and expression of Th2 cytokines in the CNS has been associated with protection from EAE (11–13). B7-1 and B7-2 are believed to differentially activate Th1 and Th2 cells in vitro (14) and in vivo (15, 16), although this idea is controversial (17, 18). In addition, blocking B7-1 or B7-2 has been shown to have differential effects on autoimmune responses in vivo depending upon the disease model studied (15, 19–21). Thus, treating proteolipid protein (PLP)-induced EAE with Ab to B7-1 reportedly prevents disease and promotes the development of Th2 cells, while treatment with anti-B7-2 resulted in the production of effector cells of a Th1 phenotype and increased disease severity (15). In contrast, blocking B7-1 worsened diabetes in nonobese diabetic mice (21), while B7-2 blockade was beneficial.

CTLA4 is a CD28 homologue that is expressed after T cell activation, interacts with B7-1/B7-2, and delivers a negative signal that terminates immune responses (22, 23). Gene knockout mice that lack CTLA4 develop severe autoimmunity and lymphoproliferative disorders and die soon after birth (24, 25). Interestingly, blocking CTLA4 in vivo has been shown to worsen autoimmune responses (26, 27) and prevent the induction of peripheral tolerance (28). These studies point out the complexity of costimulatory signaling in the initiation and maintenance of the autoimmune response in vivo.

There are data that indicate that there are differences in the expression patterns of B7-1 and B7-2 in vitro (29, 30) and in vivo (31). The patterns and kinetics of costimulatory molecule expression in the target organ during autoimmune responses have not been investigated. Such studies are essential for a better understanding of autoimmune responses in vivo and for the development of specific immunotherapeutic strategies. In this report, we investigated the expression patterns of costimulatory molecules, their ligands, and cytokines in the CNS during the natural course of
relapsing murine EAE, an experimental model of the human disease multiple sclerosis.

Materials and Methods

**Mice**

Female (PLJ × SJL)F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 wk of age; the mice were used at 10 to 12 wk of age.

**EAE induction**

(PLJ × SJL)F1 mice were immunized s.c. in the flank with 100 μg of mouse myelin basic protein (MBP) that had been prepared as described previously (32) and emulsified in an equal amount of CFA containing 2 mg/ml Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI). The animals received 200 ng of pertussis toxin i.p. (List Biologic Laboratories, Campbell, CA) at 24 h postimmunization. The scoring of clinical disease was performed daily as described previously (11, 33). In this model, animals typically develop acute disease by days 8 to 12 postimmunization. In our study, the disease incidence was 100% and the day of onset was day 10.78 ± 0.3 (mean ± SE from 28 mice in several experiments). Onset was followed by a clinical remission and then by one or more relapses. Figure 1 shows the time course of the disease in individual mice as they were euthanized for one study. The mean day of onset in this experiment was 10.73 ± 0.36, and the incidence was 100%.

**Antibodies**

The following Abs were obtained from PharMingen (San Francisco, CA); anti-glutamate receptor (N-methyl-D-aspartate receptor 1); anti-glia fibrillary acidic protein (GFAP); purified rat IgG1, IgG2a, and IgG2b; purified hamster IgG; FITC-rat anti-mouse CD11b; FITC-rat anti-mouse CD45R/B220; FITC-rat anti-mouse B7-2/2D10; FITC-rat anti-mouse B7-1/1G10; rat anti-mouse CD40; rat anti-mouse IFN-γ/R4–6A2; rat anti-mouse IL-4/BVD4-1D11; rat anti-mouse IL-10/IES5-2A5, rat anti-mouse IL-12/CI7.8; and rat anti-mouse TNF-α/MP6-XT22. Anti-TGF-β was obtained from R&D Systems (Minneapolis, MN), and rat anti-mouse macrophage clone F4/80 (Caltag, South San Francisco, CA), hamster anti-mouse CTLA4, rat anti-mouse CD80 (clone 16-10A) and CD86 (clone GL1) were obtained from PharMingen. Biotinylated swine antirabbit Ig was obtained from Dako (Glostrup, Denmark), while biotinylated anti-mouse IgG and biotinylated anti-rat IgG were purchased from Vector Labs (Burlingame, CA).

**Immunohistology**

Spinal cords were collected at various time points after immunization from three to eight mice in each experimental group: on days 0, 4, and 7 (before clinical signs of disease); between days 11 and 14 (during the first attack and peak of the disease); on day 18 (remission phase); and between days 26 and 29 (relapses) (Fig. 1). Nonimmunized and CFA-immunized mice served as controls. Spinal cord tissues were embedded in OCT, quick-frozen in liquid nitrogen, and kept at −70°C until sectioning. Cryostat sections (14 μm) of spinal cords were fixed with acetone or 4% paraformaldehyde and then labeled with the Ab of interest. The sections were stained using the avidin-biotin technique (Vectastain Elite kit; Vector Labs), visualized with diamino-benzidine (Vector Labs), and counterstained in hematoxylin. Isotype-matched Ig and omission of the primary Ab served as negative controls. Each specimen was evaluated at three different levels of sectioning or more. The whole tissue section (a longitudinal spinal cord section) was evaluated for a given cellular marker at ×40 magnification, and the results were expressed as the mean number of labeled cells per 100 mm² of spinal cord tissue. The evaluation of cytokine expression was performed semiquantitatively because of the tendency of cytokines to diffuse around cells. The following arbitrary scale was used:

**FIGURE 1.** Clinical course in mice immunized with MBP. Mice were euthanized at various time points during the disease. Each panel shows the disease course in three individual mice that had been euthanized at the peak of disease (top panel), during remission (middle panel), and during relapse (bottom panel). The x-axis represents the days after immunization with MBP/CFA. The y-axis represents the disease grade. Scoring was performed as follows: grade 0 = normal; grade 1 = floppy tail; grade 2 = hind limb paralysis; grade 3 = incontinence; grade 4 = quadriplegia; grade 5 = death.
CD40L expression according to the Kruskal-Wallis test.  

p = 0.001 for CD40, p = 0.002 for B7-1, and p < 0.05 for CD40L expression according to the Kruskal-Wallis test.

Preparation of cells from CNS and lymphoid tissue for FACS staining and analysis

Mice were sacrificed on days 10, 18, and 26 postimmunization as described above. The spleen, lymph node, spinal cord, and brain tissues were dissected and collected in RPMI 1640 media containing 10% heat-inactivated FCS. Tissues were minced using a tissue stringer; CNS tissue was cut in pieces with two scalpels. The cells were mechanically dissociated by trituration in medium using a long-necked Pasteur pipette. Cells were then washed three times by centrifugation at 125 g for 10 min; viable cells were counted and then labeled with direct or indirect fluorescent Abs for 30 min and washed three times. The cells were fixed with 4% ice-cold paraformaldehyde for 10 min, and then the samples were analyzed by two-color flow cytometry on a FACS-scan (Becton Dickinson, Mountain View, CA).

The data were analyzed using CellQuest software program.

Statistical analysis

The nonparametric Kruskal-Wallis test for multiple comparisons was used to analyze the immunohistochemistry data.

Results

B7-2 expression

B7-2 is the major CD28 ligand expressed early during an immune response (2, 29, 30), but little is known about the kinetics of the tissue-specific expression of B7-2 during the course of an autoimmune disease. Here, we studied the expression of B7-2 in the CNS in EAE and its correlation with clinical disease and in situ cytokine production. We show that B7-2 is detectable in the CNS as early as day 4 postimmunization (i.e., at the time of cellular infiltration and before the onset of clinical signs of disease) (see Fig. 2). B7-2 expression is significantly up-regulated during the acute phase of the disease and during relapses (Figs. 2 and 3). Furthermore, the kinetics of B7-2 expression coincided with the expression of IL-12 and TNF-α in the CNS (Table I). Interestingly, IFN-γ was present in the inflammatory infiltrates only during clinical attacks but not before clinical symptoms or during recovery (Table I).

FACS staining and analysis of cells isolated from the CNS confirmed the up-regulation pattern of B7-2, with a 59.5% and 51.3% increase in B7-2 expression during acute disease and relapses, respectively. Interestingly, B7-2 was not detectable during remission, although inflammatory infiltrates were still present in the CNS (Fig. 4), indicating a down-regulation of B7-2 expression. We also detected B7-2 expression on CNS resident cells such as microglia and astrocytes. Astrocytes were double-stained for GFAP and B7-2; as shown in Figure 5A, there was an up-regulation of B7-2 expression on astrocytes (58.6%) during acute disease but not during remission.

B7-1 expression

There was no detectable staining for B7-1 in the CNS during the preclinical or acute phases of EAE. However, B7-1 was significantly up-regulated in the CNS during clinical remission (Figs. 2 and 3). Interestingly, B7-1 was also expressed by neurons during remission (Figs. 5B and 6). The neuronal expression of B7-1 was confirmed using two different Abs, a rat anti-mouse Ab and a hamster anti-mouse Ab. To confirm these results, CNS tissue was dispersed into single-cell suspensions, and two-color flow cytometry was performed. FACS analysis confirmed the lack of B7-1 expression on resident or infiltrating CNS cells during the preclinical and acute phases of EAE (Fig. 4). B7-1 expression was demonstrated on neurons and astrocytes (Fig. 5) and on infiltrating cells during clinical remission (Fig. 4). In the periphery, animals immunized for EAE showed an increase in B7-1 expression in the splenocytes on day 10 postimmunization compared with naive controls. Surprisingly, B7-1 expression on T cells was decreased at a time when the total expression of B7-1 by splenocytes was increased (Fig. 7). The total number of T cells in the spleen was unchanged; since there is no B7-1 expression in the CNS during this period, a migration of B7-1-expressing T cells appears unlikely.

CD28 expression

We studied the expression of CD28 in the CNS and its correlation to disease activity. We show that CD28 is expressed on cells that infiltrate the CNS as early as day 7 postimmunization. The number of CD28-expressing cells increased significantly during clinical attacks concomitant with an up-regulation of B7-2 expression in inflammatory infiltrates (Figs. 2 and 3). Furthermore, the peak of CD28 expression in the CNS correlated with the increase in Th1 cytokine expression (Table I).

CTLA4 expression

Unlike CD28, CTLA4 is not constitutively expressed on the cell surface of T cells but is detectable early after T cell activation (22, 34). As shown in Figures 2 and 8, we did not detect any constitutive CTLA4 expression in the CNS. However, CTLA4-expressing cells were seen in perivascular infiltrates by day 4 postimmunization. CTLA4 expression was up-regulated during acute disease, peaked during remission, and persisted during relapses.
Expression of CD40 and CD40L

We found that CD40-expressing cells infiltrate the CNS as early as day 4 postimmunization. CD40 expression increased significantly during acute disease and subsequent relapses and decreased during remission (Figs. 2 and 3). Furthermore, CD40L expression on infiltrating cells increased during acute disease and peaked during relapses (Figs. 2 and 8). The appearance of CD40 in the CNS correlated with the expression of the inflammatory cytokines IL-12, IFN-γ, and TNF-α (Table I).

Cytokine expression in the CNS

There is evidence that costimulatory molecules may differentially regulate Th cytokines (14), and that cytokines may regulate the expression of costimulatory molecules during immune responses in vitro (30, 35). We investigated the relationship between costimulatory molecules and cytokine expression in the CNS of animals with relapsing EAE. We found that the expression of IL-12, IFN-γ, and TNF-α correlated closely with clinical signs; IL-12 and TNF-α expression was noted before clinical onset, and all three cytokines peaked during acute disease and during relapses (Table I and Fig. 9). In contrast, Th2 cytokines (IL-4 and IL-10) were constitutively expressed in the spinal cord; their level of expression did not correlate with disease activity or recovery (data not shown). Interestingly, TGF-β expression peaked during clinical remission (Table I and Fig. 9). In addition, neurons expressed TGF-β during remission (Fig. 6). The expression of TGF-β by

Table I. Kinetics of cytokine expression during the course of EAE

<table>
<thead>
<tr>
<th>Days Postimmunization</th>
<th>IFN-γ</th>
<th>IL-12</th>
<th>TNF-α</th>
<th>TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (naive)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4 (before clinical onset)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7 (before clinical onset)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>13 (peak of acute phase)</td>
<td>+++</td>
<td>++</td>
<td>+++++</td>
<td>++</td>
</tr>
<tr>
<td>18 (remission)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>28 (relapses)</td>
<td>+</td>
<td>+++</td>
<td>+++++</td>
<td>+</td>
</tr>
</tbody>
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* Each group consists of three to eight mice.
neurons occurred simultaneous to B7-1 expression by these cells (Figs. 5A and 6) and suggests a potential role for neuronal cells in down-regulating the immune response in vivo.

Discussion

B7-2 is the major CD28 ligand expressed early during an immune response (2). Although several studies demonstrate that an up-regulation of B7-2 precedes B7-1 expression on B cells and dendritic cells cultured in vitro (29, 30, 36), few studies have examined the expression of these cell surface molecules during the course of an immune response in vivo (31). In particular, the kinetics of expression of these molecules in relation to clinical disease have not been investigated. In this report, we show early expression of B7-2 in the CNS before the development of clinical disease; this finding is consistent with the in vitro expression data (29, 30, 36). We also observed a close correlation between the expression of B7-2 in the CNS and the clinical signs of EAE. The concordance of B7-2 expression with IL-12 and TNF-α expression suggests that B7-2-expressing APCs (for IL-12 and TNF-α) or the T cells costimulated by these APCs (for TNF-α) may be the main source of these cytokines in the CNS. Our results underline the importance of the CD28-B7-2 costimulatory pathway in the initiation of T cell priming and also emphasize the role of this pathway in the effector and maintenance phases of EAE.

Several reports suggest that there is a redundancy of B7-1 and B7-2 function in T cell activation (18, 37), while other reports suggest differential roles for B7-1 and B7-2 in T cell differentiation (15, 16). In this report, we show that although B7-1 is up-regulated during the initial phases of the immune response in the peripheral lymphoid organs, it is not expressed in the CNS tissue until the remission phase of the disease. This finding was determined by immunohistochemistry and confirmed using two different Abs for immunohistology and by FACS staining of cells isolated from CNS tissues. Furthermore, we found a decrease in B7-1 expression on T cells in the periphery at a time when total B7-1 expression in splenocytes was increased compared with naive splenocytes. Thus, B7-1 expression during the course of EAE is differentially regulated in the peripheral lymphoid tissue and in the CNS. Therefore, blocking B7-1 may have a protective (15, 19, 20) or detrimental effect (38) on EAE depending upon the site of interaction, the timing of administration, and possibly whether the reagent crosses the blood-brain barrier. In support of this hypothesis, we have recently shown, using the Lewis rat model, that the systemic administration of CTLA4IgY100F, which selectively binds and blocks B7-1, was not protective or may even worsen disease, while the administration of APCs that had been treated ex vivo with CTLA4IgY100F and encephalitogenic peptide was protective (38). In the CNS, B7-1 expression peaked during remission and was detected on neurons as well as lymphoid cells. We also show the expression of B7-1 and B7-2 by astrocytes, which is a finding that was previously reported for cultured astrocytes (39) and microglia in vitro (40–42). These cell types are also known to express MHC class II molecules and play an important role in regulating the local immune response. There is recent evidence that B7-2 on T cells is posttranslationally modified and has a reduced affinity to CTLA4 but does not bind to CD28 (43). These observations raise the

![Figure 4](http://www.jimmunol.org/)

**Figure 4.** FACS staining of cells that had been isolated from the CNS of mice at different time points after immunization. B7-2 (top panel) and B7-1 (bottom panel) staining plotted against forward scatter are shown; all cells recovered were gated. The thick outline indicates the positive staining, and the thin line histogram represents the isotype control. The percentage of positive cells is shown in each histogram.

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** FACS staining of isolated CNS cells during relapse and remission. A. Astrocytes were gated positively according to GFAP, and forward scatter is plotted against B7-2 (top panel) and B7-1 (bottom panel) expression. The thick outline indicates the positive staining, and the thin line histogram represents the isotype control. B. Staining for B7-2 (top panel) and B7-1 (bottom panel) in isolated neurons after gating for N-methyl-D-aspartate receptor 1-positive cells. The percentage of positive cells is shown in each histogram.
possibility that the B7-1 expressed by nonlymphoid cells in the CNS may be important in down-regulating the immune response through interactions with CTLA4. On the other hand, the expression of B7-1 on mononuclear infiltrates in the CNS during remission could be interpreted as a prelude to reactivation and the occurrence of a relapse. This interpretation would be more consistent with previous reports showing that a B7-1 blockade prevents clinical relapses and epitope spreading (20). It is important to point out that the type of cell expressing B7-1 and its interaction with CD28 or CTLA4 may play a crucial role in the modulation of an immune response in vivo. This might explain the apparently conflicting results seen with a B7-1 blockade in autoimmune diseases. In the EAE model in particular, B7-1 blockade was reported to exacerbate or protect from disease depending upon the timing of administration (19, 44). Our findings with B7-1 expression exclusively during remission do not agree with the findings by Miller et al. of an enhanced expression of B7-1 on mononuclear cells isolated from the CNS during acute EAE (20). The model used in that report was a PLP-induced disease in SJL mice. The priming Ag and the genetic background of the mouse strain may contribute to these differences.

The engagement of CTLA4 with B7 molecules transduces a negative signal which leads to the inactivation of T cells (22–25, 45). In vitro, CTLA4 is expressed on T cells within 24 h of activation and is down-regulated after a few days (22, 34). Since all T cells are not activated synchronously, the number of T cells expressing CTLA4 at a given time point is lower than that of cells expressing CD28. The absence of CTLA4 staining in the CNS during the preclinical stages suggests that activated cells down-regulate their CTLA4 expression as soon as they penetrate the CNS, and expression of CTLA4 is seen only after T cells are reactivated locally. The peak expression of CTLA4 correlated with the appearance of B7-1 expression in the CNS, suggesting a potential regulatory role for B7-1-CTLA4 interaction, as discussed above (38).

Our immunohistologic data confirm an important role of CD40L-CD40 in the acute and relapsing phases of EAE. CD40 is constitutively expressed on B cells, dendritic cells, and monocytes/macrophages (46), while CD40L is transiently expressed on activated CD4+ T cells (47). An interaction of CD40 with its ligand is believed to be involved in T cell activation (48, 49) and is essential for B cell responses against thymus-dependent Ags (3, 50, 51). More recently, it was shown that CD40L is required for T cell costimulatory activation in PLP-mediated EAE (5), and that

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Photomicrograph of immunoperoxidase staining of cryostat sections of mouse spinal cords. Shows motor (a) and sensory (dorsal root ganglia) (c) neurons expressing B7-1 during remission. b, Astrocytes with typical morphology expressing B7-2 during exacerbations. d, TGF-β staining of sensory neurons during remission. Positive-staining cells are brown.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** FACS staining of splenocytes for B7-1 expression during acute disease. A, The whole splenocyte population was gated, and forward scatter is plotted against B7-1 expression. The thick outline indicates the positive B7-1 staining during acute disease, the thin outline indicates the B7-1 staining in naive animals, and the filled histogram represents the isotype control. B shows similar staining for T cells, which were gated according to CD3 positivity.
anti-CD40L protects against clinical EAE (52). Interestingly, in the study by Grewal et al. (5) B7-1 costimulation appears to mediate the effect of CD40L-CD40 costimulation in EAE. CD40-CD40L interaction is critical for IL-12 secretion by macrophages and dendritic cells (49, 53), which may explain the ability of anti-CD40L mAb to inhibit disease in animal models that are dependent upon the generation of a cell-mediated immune response.

As previously reported, the expression of Th1 cytokines correlated with disease activity in EAE (11, 54, 55). We found that TGF-β expression correlated with recovery, which is consistent with the role of this factor in recovery from EAE (11, 56–58). This is the first report describing the simultaneous expression of TGF-β and B7-1 by neurons in EAE during remissions. This finding suggests that neurons may play a role in the regulation of local inflammation by producing TGF-β and by expressing B7-1 that can potentially interact with CTLA4 on the surface of T cells to induce anergy or the apoptosis of encephalitogenic T cells. Neuronal staining for B7-1 could be attributed to cross-reactivity with a neuronal Ag; however, the expression of B7-1 was confirmed using two different Abs, which decreases the likelihood of cross-reactivity, and the fact that staining was seen only during remission also makes this possibility unlikely. Neurons are not known to express MHC molecules, and consequently may not be able to provide signal 1 to T cells. However, signal 1 and signal 2 can be provided by two different cells in what has been termed trans-costimulation, in which one cell type provides signal 1, and another cell type provides signal 2 (59, 60). The TGF-β detected in neurons is probably in a latent form, since latent TGF-β is normally activated after being secreted (61). Thus, we cannot determine by immunohistology whether TGF-β from neurons will end up being hydrolyzed to its active form. The factors that regulate the expression of costimulatory molecules and/or the secretion of suppressive cytokines or growth factors by neurons must be investigated.

In summary, this is the first demonstration of the kinetics of expression of costimulatory molecules and their ligands in vivo during the course of an autoimmune response characterized by clinical remissions and relapses. Further understanding of the mechanisms that regulate the local expression of costimulatory molecules and their ligands is essential for designing novel and specific therapies to block T cell activation in tissue-specific autoimmune diseases in humans.
References


