

## Decreased Dependence of Myelin Basic Protein-reactive T Cells on CD28-mediated Costimulation in Multiple Sclerosis Patients

### A Marker of Activated/Memory T Cells

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#### Abstract

Although multiple sclerosis (MS) patients and healthy individuals have similar frequencies of myelin basic protein (MBP)-specific T cells, the activation state of these cells has not been well characterized. Therefore, we investigated the dependence of MBP-reactive T cells on CD28-mediated costimulation in MS patients, healthy controls, and stroke patients. MBP-reactive T cells from healthy controls and stroke patients failed to proliferate efficiently when costimulation was blocked using anti-CD28, consistent with a naive T cell response. In contrast, MBP-specific T cell proliferation was not inhibited, or was only partially inhibited when CD28-mediated costimulation was blocked in MS patients. Blockade of CD28 failed to inhibit tetanus toxoid-specific T cell proliferation in both the controls and MS patients, demonstrating that memory cells are not dependent on CD28-mediated costimulation. Limiting dilution analysis indicated that the frequency of MBP-reactive T cells was significantly decreased in healthy controls compared with MS patients when CD28-mediated costimulation was blocked. These data suggest that MBP-reactive T cells are more likely to have been activated *in vivo* and/or differentiated into memory T cells in MS patients compared with controls, indicating that these cells may be participating in the pathogenesis of MS. (*J. Clin. Invest.* 1998. 101:725–730.) **Key words:** costimulation • immunological memory • multiple sclerosis • myelin basic protein • T cells

#### Introduction

Multiple sclerosis (MS)<sup>1</sup> is a demyelinating disease of the central nervous system (CNS), pathologically characterized by

perivascular mononuclear cell infiltrates preceding myelin loss. The cause of MS is unknown, but it has been postulated that the myelin damage is immune-mediated, either secondary to a viral infection or a direct autoimmune process. The role of myelin-reactive T cells in the pathogenesis of MS is uncertain. MS patients and controls have been shown to have T cells reactive to a number of myelin antigens, making it difficult to establish a relationship between myelin reactivity and the presence of disease (1–4). Most studies attempting to differentiate characteristics of myelin-specific T cells in MS patients and controls have focused on the trimolecular complex (TCR, peptide and MHC). However, restricted usage of particular elements of the trimolecular complex, observed in the acute stage of experimental models of MS (experimental allergic encephalomyelitis), has not been consistently observed in MS patients (1–4). The state of activation or differentiation of myelin-reactive T cells has not been well-characterized. Myelin-specific T cells with a mutation in the HPRT gene have been found at a higher frequency in MS patients, suggesting that these T cells have a more extensive replicative history and have had the opportunity to mutate (5, 6). In addition, the frequency of myelin-specific T cells expressing the IL-2R, a marker of activation, was shown to be higher in MS patients (7).

New insights into the requirements for T cell activation and peripheral tolerance induction may provide a means of identifying myelin-reactive T cells that have responded to antigens *in vivo*, enabling one to characterize potentially pathogenic T cells. Activation of naive T cells requires two signals. Signal one, which is antigen-specific, involves engagement of the TCR by peptide bound to MHC. Signal two, termed costimulation, is usually delivered by CD80 (B7-1) or CD86 (B7-2) on the APC to CD28 on the T cell. Once a T cell is primed, it is less dependent on the second signal (8). Engagement of the TCR on naive T cells in the absence of costimulation results in anergy *in vitro* (9, 10). It is believed that this anergy may be an important peripheral tolerance mechanism, preventing activation of autoreactive T cells that are not deleted in the thymus (11). Recently it was shown that CD152 (CTLA-4), a counter-receptor for B7-1 and B7-2 known to downregulate the immune response, may play a critical role in extrathymic tolerance induction *in vivo* (12–16). In addition, memory T cells have been shown to be less dependent on CD28-mediated costimulation for reactivation than are naive T cells (17). To address

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1. *Abbreviations used in this paper:* CNS, central nervous system; MBP, myelin basic protein; MS, multiple sclerosis; TT, tetanus toxoid.

the paradox that patients with MS and controls have similar frequencies of circulating myelin basic protein (MBP)-specific T cells, we examined the ability of unmanipulated MBP-reactive T cells to proliferate when CD28-mediated costimulation was blocked in MS patients, stroke patients, and healthy controls. We found that MBP-reactive T cells are less dependent on costimulation for proliferation in MS patients. Therefore, our results are consistent with the hypothesis that MBP-reactive T cells from MS patients have been previously activated *in vivo* and/or have more extensive differentiated memory effector cells than do MBP-reactive T cells from controls.

## Methods

**Patients and controls.** Eleven patients with clinically definite MS were used in this study. All MS patients had established disease (diagnosed at least 1 yr before entering the study) ranging from a mild relapsing/remitting course to long-term chronic progressive disease. The age range of the patients was 25–62 yr with a mean age of 42 yr; 10/11 were HLA DR15<sup>+</sup>. Eight control subjects with no significant medical history, and of similar age range (27–50 yr; mean age = 39 yr) and HLA type (6/8 were HLA DR15<sup>+</sup>) as the MS patients were used in this study. Seven patients who were diagnosed at Barnes-Jewish Hospital with cerebral infarcts (stroke) were recruited for this study.

**Peripheral blood mononuclear cells.** The MS patients and healthy controls were leukaphoresed in accordance with a protocol approved by Washington University Human Studies Committee (IRB). Under the same protocol, 60–80 cc of whole blood was collected via venipuncture from the stroke patients. PBMC were purified using Ficoll-Paque™ Plus (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer's protocol. The PBMC from the whole blood were used fresh. Cryopreserved or fresh PBMC were used from the leukaphereses.

**Reagents.** Bovine MBP was purchased from Sigma Chemical Co. (St. Louis, MO). Tetanus toxoid (TT) was purchased from Accurate Chemical and Science Corp. (Westbury, NY). mAb 9.3 (mouse IgG2a) recognizes human CD28 (18). mAb F23.1 (mouse IgG2a) was used as an isotype control for anti-CD28. CTLA-4Ig and a control fusion protein hlgG1 (10) were a gift from Gary Gray (Genetics Institute, Cambridge, MA).

**Costimulation-independent lymphocyte proliferation assay.** Human PBMC were plated at  $2.5 \times 10^5$  cells/well into 96-well, flat-bottomed plates in RPMI-1640 with 5% human AB serum. The control wells consisted of 48 replicates with no antigen and 5  $\mu$ g/ml of anti-CD28 (mAb 9.3), control IgG2a (mAb F23.1), CTLA-4Ig, or control chimeric Ig. For MBP- (10  $\mu$ g/ml) and TT- (10  $\mu$ g/ml) specific proliferation assays, 96 or 192 replicate wells were plated with anti-CD28, control IgG2a, CTLA-4Ig, and control chimeric Ig at 5  $\mu$ g/ml. On the sixth day of culture, 0.25  $\mu$ Ci [<sup>3</sup>H]thymidine was added to each well. The cells were harvested on the seventh day, and cpm/well was determined. A positive well was defined as a well with antigen and B7/CD28 antagonist or control that had a stimulation index > 2 and cpm > 2 SD above the mean of the no-antigen control wells.

**Frequency determination of costimulation-dependent T cells.** PBMC were plated at  $2 \times 10^5$ ,  $1 \times 10^5$ , and  $5 \times 10^4$  into 96-well flat-bottomed plates. Irradiated autologous PBMCs were added to the wells such that each well contained  $2 \times 10^5$  PBMCs. MBP (10  $\mu$ g/ml), TT (10  $\mu$ g/ml), or no antigen was added to 48 or 96 replicate wells of each cell concentration with 5  $\mu$ g/ml anti-CD28 or control IgG2a. On the sixth day of culture, 0.25  $\mu$ Ci [<sup>3</sup>H]thymidine was added to each well. The cells were harvested on the seventh day, and cpm was determined. A positive well was defined as having cpm > 2 SD above the mean of the no-antigen control. The percentage of negative wells for each cell concentration was determined, and  $\chi^2$  minimization was used to estimate the frequency of responding T cells in the PBMC (19).

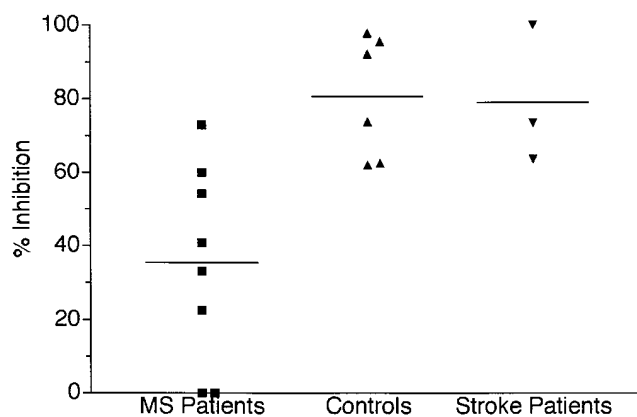
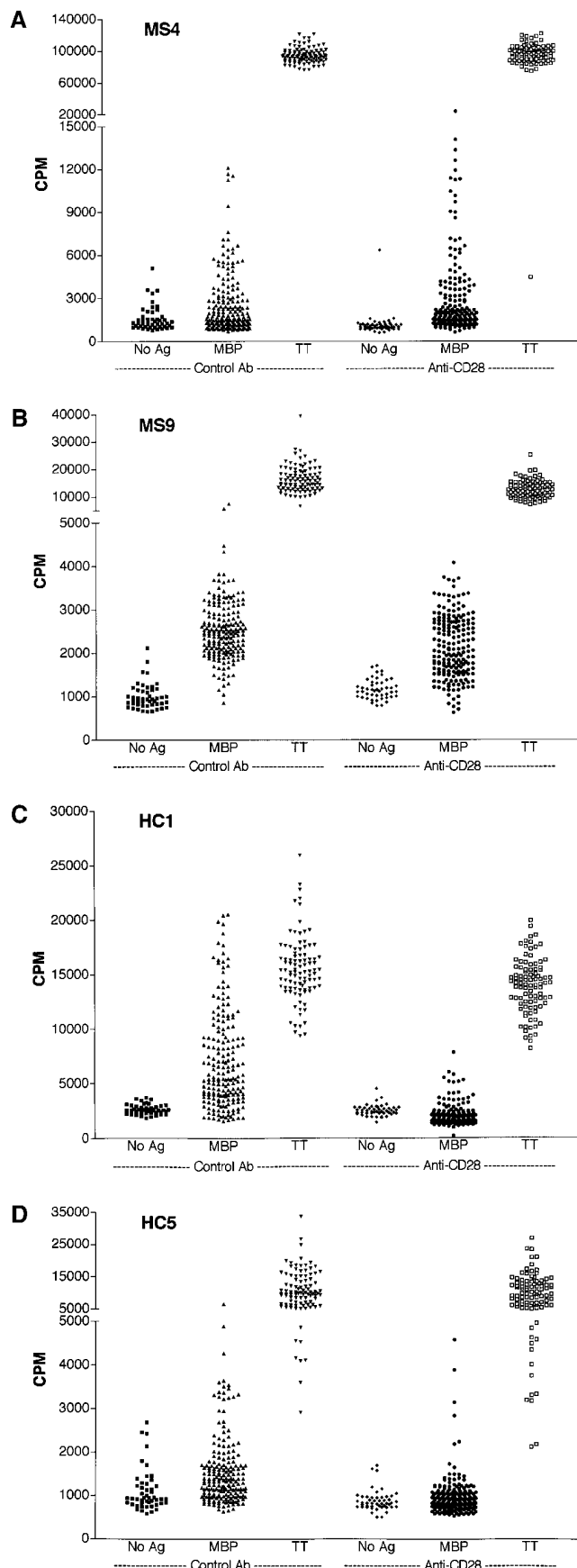
## Results

**Examination of costimulatory requirements of MBP-specific T cells in MS patients, stroke patients, and controls.** To determine if MBP-reactive T cells from MS patients have a phenotype consistent with T cells that have been activated *in vivo*, we examined the dependence of unmanipulated MBP-reactive T cells on CD28-mediated costimulation for proliferation. PBMCs instead of T cell lines or clones were used in these experiments because *in vitro* restimulation of antigen-specific T cells results in T cells that are independent of CD28-mediated costimulation regardless of their *in vivo* activation state (8). Anti-CD28 (mAb 9.3) can be stimulatory in solution when anti-CD3 is used to cross-link the TCR (18). However, soluble mAb 9.3 blocks B7 costimulation from APC-delivering signal one, while costimulation delivered *in trans* by the soluble mAb 9.3 does not efficiently activate antigen-specific T cells (11, 20). Fig. 1 shows the cpm of each well of the lymphocyte proliferation assays using PBMCs from two MS patients and two

**Table I. Myelin Basic Protein-reactive T Cells are Less Dependent on Costimulation for Proliferation in Multiple Sclerosis Patients**

|                                    | Myelin basic protein |           | Tetanus toxoid |           |
|------------------------------------|----------------------|-----------|----------------|-----------|
|                                    | Control IgG2a        | Anti-CD28 | Control IgG2a  | Anti-CD28 |
| <b>Multiple sclerosis patients</b> |                      |           |                |           |
| MS1                                | 97/192*              | 75/192    | 96/96          | 95/96     |
| MS2                                | 1/192                | 0/192     | 0/96           | 0/96      |
| MS3                                | 164/192              | 75/192    | 96/96          | 96/96     |
| MS4                                | 26/192               | 31/192    | 96/96          | 96/96     |
| MS5                                | 0/192                | 0/192     | ND             | ND        |
| MS6                                | 63/192               | 17/192    | 96/96          | 96/96     |
| MS7                                | 130/192              | 87/192    | 96/96          | 96/96     |
| MS8                                | 1/192                | 2/192     | 96/96          | 96/96     |
| MS9                                | 130/192              | 77/192    | 96/96          | 96/96     |
| MS10                               | 13/192               | 17/192    | ND             | ND        |
| MS11                               | 20/192               | 8/192     | ND             | ND        |
| <b>Healthy controls</b>            |                      |           |                |           |
| HC1                                | 103/192              | 8/192     | 96/96          | 96/96     |
| HC2                                | 0/192                | 0/192     | 96/96          | 95/96     |
| HC3                                | 129/192              | 49/192    | ND             | ND        |
| HC4                                | 0/192                | 0/192     | ND             | ND        |
| HC5                                | 16/192               | 6/192     | 96/96          | 96/96     |
| HC6                                | 47/192               | 1/192     | 1/96           | 0/96      |
| HC7                                | 130/192              | 34/192    | ND             | ND        |
| HC8                                | 67/192               | 3/192     | 77/96          | 81/96     |
| <b>Stroke patients</b>             |                      |           |                |           |
| SP1                                | 0/192                | 0/192     | ND             | ND        |
| SP2                                | 72/192               | 19/192    | ND             | ND        |
| SP3                                | 1/192                | 0/192     | ND             | ND        |
| SP4                                | 11/192               | 4/192     | ND             | ND        |
| SP5                                | 0/96                 | 2/96      | ND             | ND        |
| SP6                                | 0/96                 | 0/96      | ND             | ND        |
| SP7                                | 3/96                 | 0/96      | ND             | ND        |

\*The number of positive wells/the number of total wells plated. A well was considered positive if the SI was > 2 and the cpm were > 2 SD above the mean of the control wells. ND, Not done.



**Figure 2.** Blockade of CD28-mediated costimulation did not efficiently inhibit proliferation of MBP-reactive T cells in most MS patients. The percent inhibition of the number of positive wells in lymphocyte proliferation assays in the presence of anti-CD28 was determined for the patients and controls who had a response rate > 3% with the control antibody (Table I). A two-tailed *t* test indicated a significant difference between the MS patients and controls (MS patients vs. controls,  $P = 0.0036$ ; MS patients vs. stroke patients,  $P = 0.0317$ ; controls vs. stroke patients, NS).

healthy controls. Table I summarizes the data from 11 MS patients, 8 healthy controls, and 7 stroke patients. As previously observed, T cells from control subjects proliferated to MBP as well as T cells from the MS. However, when CD28 was blocked with anti-CD28, the number of positive wells decreased significantly for the healthy controls and stroke patients. In contrast, MBP-specific proliferation was not blocked, or was only partially blocked in the presence of anti-CD28 in the MS patients. Analysis of the patients and controls that had a response rate to MBP with the control antibody > 3% in Table I indicated that there was a significant difference in the need for CD28-mediated costimulation of MBP-reactive T cells between the MS patients and controls (Fig. 2; MS vs. controls,  $P = 0.0036$ ; MS vs. stroke patients,  $P = 0.0317$ ; controls vs. stroke patients, NS). The mean percent inhibition with anti-CD28 was 35% for the MS patients, 80% for the healthy con-

**Figure 1.** CD28 blockade inhibits MBP-specific lymphocyte proliferation in healthy controls, but not in MS patients. Lymphocyte proliferation assays were done with  $2.5 \times 10^5$  PBMCs/well using no ag (48 replicates), MBP (10  $\mu\text{g/ml}$ ; 192 replicates), or TT (10  $\mu\text{g/ml}$ ; 96 replicates) in the presence of anti-CD28 (mAb 9.3 at 5  $\mu\text{g/ml}$ ) or a control ab (mAb F23.1 at 5  $\mu\text{g/ml}$ ). Each symbol represents the cpm of a well. (A) MS4. For the no ag + control ab wells, mean = 1599.8 and SD = 1729.7 (cpm > 5059.2 is positive). For the no ag + anti-CD28 wells, mean = 1165.8 and SD = 1583.9 (cpm > 4333.6 is positive). (B) MS9. For the no ag + control ab wells, mean = 1011.0 and SD = 592.9 (cpm > 2196.8 is positive). For the no ag + anti-CD28 wells, mean = 1167.3 and SD = 450.6 (cpm > 2334.6 is positive). (C) HC1. For the no ag + control ab wells, mean = 2636.4 and SD = 884.5 (cpm > 5272.8 is positive). For the no ag + anti-CD28 wells, mean = 2523.5 and SD = 1009.5 (cpm > 5047.0 is positive). (D) HC5. For the no ag + control ab wells, mean = 1112.8 and SD = 966.8 (cpm > 3046.4 is positive). For the no ag + anti-CD28 wells, mean = 881.5 and SD = 482.7 (cpm > 1846.9 is positive). See Table I for total number of positive wells.

trols, and 79% for the stroke patients. The observation that MBP-reactive T cells from MS patients were less dependent on costimulation for proliferation suggests that they were activated or differentiated memory T cells.

The role of costimulation in reactivating memory T cells is unclear. To address whether memory T cells are dependent on CD28-mediated costimulation for proliferation, we analyzed TT-specific lymphocyte proliferation in the MS patients and healthy controls. Addition of anti-CD28 to TT-specific lymphocyte proliferation assays failed to alter the response rate in either the MS patients or healthy controls using culture conditions identical to the MBP-specific assays (Fig. 1 and Table I). A dose response assay using 6.25–100  $\mu\text{g/ml}$  of anti-CD28 did not alter the response rate of TT-specific lymphocyte proliferation (data not shown). Since most individuals have been immunized to tetanus, but are not exposed to this antigen such that one would maintain a significant population of activated T cells, it is probable that most TT-specific T cells have a memory phenotype *in vivo*. Thus, this data supports the hypothesis that CD28-mediated costimulation is not essential for memory T cell proliferation.

*The frequency of CD28-independent MBP-specific T cells is significantly lower in controls than MS patients.* Previous studies have shown that the frequency of MBP-reactive T cells in peripheral blood of MS patients and controls is similar (3). To verify that the number of T cells in the peripheral blood specific for MBP that are dependent on CD28-mediated costimulation for proliferation actually differs between MS patients and controls, limiting dilution analysis was performed. Lymphocyte proliferation assays were set with various concentrations of PBMC with no antigen, MBP, or TT in the presence of anti-CD28 or control IgG2a as described in Methods, and  $\chi^2$  minimization was used to estimate the frequency of responding T cells in the PBMC. In the two MS patients analyzed, the frequency of T cells responding to MBP was only slightly lower in the presence of anti-CD28 (Table II). However, this difference was not statistically significant, as the 95% confidence intervals overlapped between the control IgG2a and anti-CD28-treated wells. In contrast, there was a significant decrease (four to sixfold) in the frequency of T cells responding to MBP in the presence of anti-CD28 in the two healthy

Table II. Frequency of MBP and TT-reactive T Cells in Peripheral Blood Mononuclear Cells with CD28 Blockade

|               | MBP frequency* | TT frequency |
|---------------|----------------|--------------|
| MS3           |                |              |
| Control IgG2a | 1/178,000      | 1/96,300     |
| Anti-CD28     | 1/209,000      | 1/62,200     |
| MS9           |                |              |
| Control IgG2a | 1/1,700,000    | ND           |
| Anti-CD28     | 1/2,040,000    | ND           |
| HC1           |                |              |
| Control IgG2a | 1/2,900,000    | 1/52,200     |
| Anti-CD28     | 1/12,600,000   | 1/40,700     |
| HC5           |                |              |
| Control IgG2a | 1/805,000      | 1/260,000    |
| Anti-CD28     | 1/5,000,000    | 1/208,000    |

\*Frequency was estimated using  $\chi^2$  minimization. ND, not done.

controls. The TT-specific T cell frequency did not vary significantly in any subject when CD28 was blocked. This data confirmed that the actual number of T cells capable of responding to MBP in the absence of costimulation is significantly lower in healthy controls, suggesting that these cells are naive.

*Blockade of B7-1 and B7-2 by CTLA-4Ig inhibits proliferation similarly to anti-CD28.* B7-1 and B7-2 on APC deliver the costimulatory signal to T cells via CD28. To verify that anti-CD28 was blocking proliferation through the CD28/B7 pathway, a chimeric molecule (CTLA-4Ig) that binds B7-1 and B7-2 and effectively blocks costimulation, was used (10, 21). We compared the ability of this molecule and a control chimeric Ig to inhibit MBP and TT-specific T cell proliferation in an MS patient (MS6). CTLA-4Ig inhibited MBP-specific proliferation by 38% compared with 47% by anti-CD28 in an MS patient (Fig. 3). Similarly, CTLA-4Ig did not alter the TT-specific proliferative response. This result confirms that blocking CD28 interaction with B7-1 and B7-2 on the T cell or APC results in only partial inhibition of MBP-specific proliferation in MS patients.

## Discussion

It has been difficult to determine if myelin-reactive T cells play a role in the pathogenesis of MS because healthy individuals also have myelin-reactive T cells at similar frequencies to MS patients (3). One possible explanation for the similar frequencies of MBP-reactive T cells is that the vast regulatory networks of the immune system may downregulate the autoimmune process in the CNS, inhibiting our ability to observe

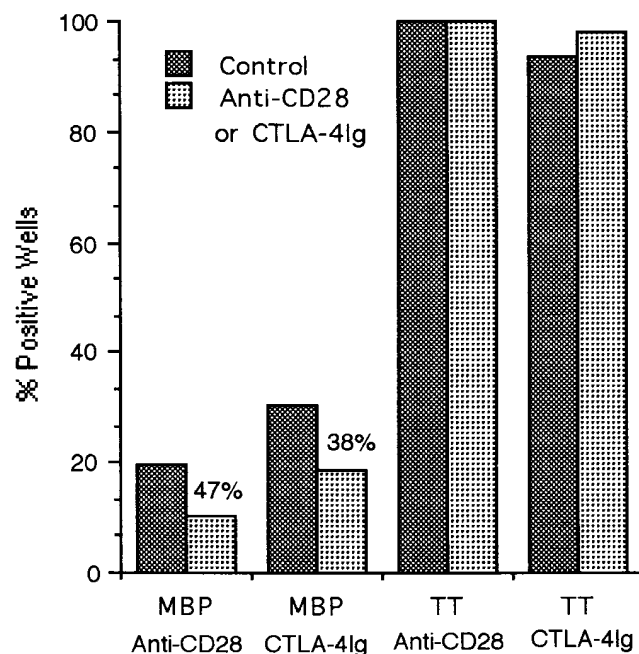


Figure 3. Blockade of B7-1 and B7-2 with CTLA-4Ig inhibited proliferation similarly to anti-CD28. CTLA-4Ig or a chimeric control Ig was added to MBP and TT-specific lymphocyte proliferation assays using PBMC from MS6. The percent inhibition of positive wells is shown for both anti-CD28 and CTLA-4Ig for the MBP-specific response.

changes in frequency in the peripheral blood of MS patients. However, if myelin-reactive T cells have encountered antigens *in vivo*, their state of activation should be different. Therefore, we investigated the dependence of MBP-reactive T cells on CD28-mediated costimulation in MS patients, healthy controls, and stroke patients to determine if potentially pathogenic T cells with an activated or memory phenotype could be differentiated from naive T cells based on costimulation requirements. We found that MS patients have more MBP-reactive T cells capable of proliferating in the absence of B7/CD28 engagement than do healthy controls or stroke patients (Tables I and II), indicating that these cells have been activated *in vivo*.

One might expect that costimulation blockade should completely inhibit MBP-specific proliferation in the healthy controls, and conversely have no effect on proliferation of MBP-reactive T cells in MS patients if these cells play a role in MS. One explanation for the incomplete inhibition of MBP-reactive T cell proliferation in healthy controls after CD28 blockade may be due to the cross-reactivity of MBP-reactive T cells with foreign antigens. Studies have described MBP-reactive T cells that could proliferate to a variety of peptides derived from common pathogens, suggesting that these "MBP-specific" T cells may have been activated *in vivo* in controls in response to an infection, and may have never encountered MBP, which is sequestered behind an intact blood-brain barrier (22, 23). The variability of the ability of CD28 blockade to inhibit MBP-specific proliferation in MS patients may be due to the frequency and duration of blood-brain barrier breakdown. Recent studies examining MS patients by magnetic resonance imaging suggested that the underlying inflammatory process was quite dynamic (24, 25). Therefore, the percentage of previously primed myelin-reactive T cells in MS patients may depend on the extent of blood-brain barrier breakdown, myelin damage, and lesion burden, which vary significantly among patients. Since T cell activation requirements are decreased when TCR avidity is high, differences in TCR usage by MS patients may be partially responsible for the variability in costimulation requirements (26). In addition, we have previously shown that the population of MBP-reactive T cells appears to be changing during the course of disease, suggesting that naive MBP-specific T cells may be continually recruited into the CNS and activated (27). Longitudinal analysis of costimulation requirements of myelin-reactive T cells will enhance our understanding of the role of these cells in disease pathogenesis and progression.

The stroke patients represent an important control group because presumably after a cerebral infarct, macrophages that are clearing away the infarcted tissue would have the opportunity to present MBP to T cells. It has previously been shown that B7-1 is expressed in the CNS inflammatory lesions of MS patients, but not in cerebral infarcts, enhancing the opportunity for naive MBP-specific T cells to be primed in MS patients, but not in stroke patients (28). Our data, as well as the observation that stroke is not a risk factor for MS development (29), indicate that MBP-reactive T cells are not primed *in vivo* following cerebral infarct.

To verify that memory T cells are not dependent on CD28-mediated costimulation, the effect of CD28 blockade on TT-specific lymphocyte proliferation was also investigated. Most individuals have been exposed to TT via routine immunizations, and consequently have a robust recall response in stan-

dard lymphocyte proliferation assays (30). The role of costimulation in reactivating memory T cells is unclear. Studies have shown a decrease in T cell proliferation to recall antigens when the B7/CD28 pathway is blocked using high concentrations of antagonists (30). However, these studies fail to completely inhibit memory T cell function, suggesting that reactivation of memory T cells was not costimulation-dependent, but may be enhanced with costimulation. In lymphocyte proliferation assays using identical culture conditions as the MBP-specific assays, CD28 blockade did not alter the response rate of TT-specific proliferation, indicating that memory T cells proliferate in the absence of costimulation (Table I). Because of the higher frequency of TT-reactive T cells, the assay may not have been sensitive to partial inhibition. However, limiting dilution analysis clearly demonstrated that the frequency of proliferating TT-reactive T cells did not change significantly when the B7/CD28 pathway was blocked (Table II).

Studies in experimental allergic encephalomyelitis, an animal model for MS, have demonstrated that blocking costimulation during disease induction inhibits the onset of disease (31). However, once disease has been induced, costimulatory blockade has no effect. Interestingly, blockade of B7-1 during remissions in experimental allergic encephalomyelitis resulted in reduction of subsequent disease relapses due to inhibition of epitope spreading (32). These observations may provide the framework for therapeutic use of costimulatory antagonists in MS. Therefore, understanding the costimulatory requirements of myelin-reactive T cells in MS patients and how they change during the course of disease is essential for determining whether costimulatory blockade may be beneficial in MS treatment.

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