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Loss of Functional Suppression by $CD4^+CD25^+$ Regulatory T Cells in Patients with Multiple Sclerosis

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Abstract

$CD4^+CD25^+$ regulatory T cells contribute to the maintenance of peripheral tolerance by active suppression because their deletion causes spontaneous autoimmune diseases in mice. Human $CD4^+$ regulatory T cells expressing high levels of CD25 are suppressive in vitro and mimic the activity of murine $CD4^+CD25^+$ regulatory T cells. Multiple sclerosis (MS) is an inflammatory disease thought to be mediated by T cells recognizing myelin protein peptides. We hypothesized that altered functions of $CD4^+CD25^{hi}$ regulatory T cells play a role in the breakdown of immunologic self-tolerance in patients with MS. Here, we report a significant decrease in the effector function of $CD4^+CD25^{hi}$ regulatory T cells from peripheral blood of patients with MS as compared with healthy donors. Differences were also apparent in single cell cloning experiments in which the cloning frequency of $CD4^+CD25^{hi}$ T cells was significantly reduced in patients as compared with normal controls. These data are the first to demonstrate alterations of $CD4^+CD25^{hi}$ regulatory T cell function in patients with MS.

Key words: tolerance • autoreactive T cells • autoimmune disease • L-selectin

Introduction

Clonal deletion of self-reactive T cells in the thymus and induction of T cell anergy do not alone explain the maintenance of immunologic self-tolerance, as potentially pathogenic auto-reactive T cells are present in the periphery of healthy individuals (1, 2). Thus, other regulatory mechanisms exist to prevent autoreactive T cells from causing immune disorders. Active suppression by regulatory T cells plays a key role in the control of self-antigen–reactive T cells and the induction of peripheral tolerance in vivo (3, 4). Seminal experiments performed by Sakaguchi et al. have shown that depletion of $CD4^+CD25^+$ suppressor cells results in the onset of systemic autoimmune diseases in mice (5). Furthermore, cotransfer of these cells with $CD4^+CD25^-$ cells prevents the development of experimentally induced autoimmune diseases such as colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis (6–10). We and others have recently described a population of $CD4^+CD25^{hi}$ regulatory T cells in human peripheral blood and thymus (11–13). Human $CD4^+CD25^{hi}$ T cells, similar to the mouse $CD4^+CD25^+$ suppressor cells, are anergic to in vitro antigenic stimulation and strongly suppress the proliferation of responder T cells upon coculture (14). $CD4^+CD25^+$ T cells are among the best-characterized immunoregulatory subsets shown to prevent activation and effector function of activated responder T cells (15).

Multiple sclerosis (MS) is a chronic inflammatory disease characterized by lymphocyte infiltration and inflammation of the central nervous system (CNS) white matter. T cells recognizing myelin protein peptides are likely involved in the pathogenesis of the disease (16–18). Although autoreactive T cells are present in healthy individuals and patients with autoimmune disorders, autoreactive T cells found in patients with autoimmune disease are more easily activated as compared with those from normal subjects (19–22). This finding led us to hypothesize that either deficient generation or reduced effector function of $CD4^+CD25^{hi}$ T cells play a role in regulating the autoimmune response in patients with MS. Thus, we compared the frequency and function of $CD4^+CD25^{hi}$ T regulatory cells derived from a group of untreated patients who have relapsing/remitting (RR) MS with those from age matched healthy control subjects.

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Abbreviations used in this paper: CNS, central nervous system; MBP, myelin basic protein; MS, multiple sclerosis; RR, relapsing/remitting.

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Materials and Methods

Subjects. We enrolled 15 patients in this study with definite MS upon informed consent. This work was approved by the Institutional Review Board at the Brigham and Women's Hospital. The patients were between the ages of 25 and 57 yr, and all had RR disease with Kurtzke Expanded Disability Status Scale scores between 0 and 2.5. Patients were not treated with any immunomodulatory drugs and did not receive corticosteroids within 3 mo of study entry. 21 normal healthy donors between the ages of 24 and 57 yr and with no history of autoimmune diseases were also enrolled in the study. Three healthy subjects between the ages of 20–30 received the influenza virus vaccination, and blood was drawn at the time points outlined in the Results section.

Cell Culture Reagents. Cells were cultured in RPMI 1640 medium supplemented with 5 mM Hepes, 2 nM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (BioWhittaker), 0.5 mM sodium pyruvate, 0.05 mM of nonessential amino acids (GIBCO BRL), and 5% human AB serum (Omega Scientific Inc.) in U-bottom 96-well plates (CoStar).

Cell Purification. Human $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ T cells were separated on a FACSVantage™ SE (BD Biosciences) cell sorter. PBMCs were isolated by Ficoll-Paque (Amersham Biosciences) gradient centrifugation. Approximately 2×10^8 cells for each experiment were incubated for 1 h in the culture medium, with 300 μl anti–CD4-CyChrome (cat. no. 555348, IgG1; BD Biosciences), anti–CD25-PE (cat. no. IM0479, IgG2a; Immunotech), and a cocktail of anti-CD14 (cat. no. 30544X, IgG2a), anti-CD32 (cat. no. 30934X, IgG2b), and 20 μl anti-CD116 (cat. no. 18774B, IgM), all FITC labeled (BD Biosciences). Control PBMCs (10^6 cells each) were also stained with the aforementioned antibodies in combination with different mouse IgGs (IgG2a PE-labeled cat. no. 55574; IgG1 CyChrome-labeled cat. no. 555750; IgG1 FITC-labeled cat. no. 555748; IgG2a FITC-labeled cat. no. 555573, and IgG2b FITC-labeled cat. no. 555742; all purchased from BD Biosciences). Lymphocytes were washed and sorted according to their forward and side scatter properties, excluding large activated cells. T cell–depleted accessory cells were obtained by negative selection of PBMCs incubated with anti-CD2–coated beads (111.13; Dynal) and irradiated at 3,300 rad.

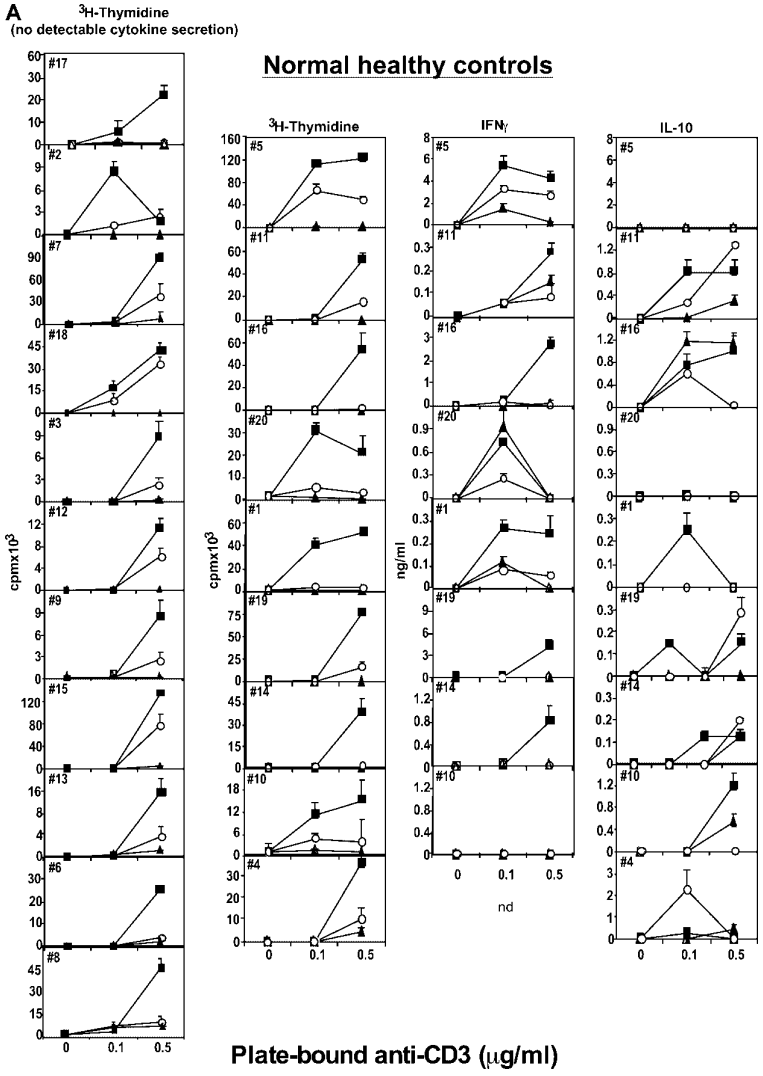

Figure 1. Human $CD4^+CD25^{hi}$ T cells mediate suppression of $CD4^+CD25^-$ responder cell proliferation. (A) The regulatory properties of $CD4^+CD25^{hi}$ T cells were examined from 21 healthy individuals or (B) from 15 patients with RR MS. $CD4^+CD25^-$ responder (■) and $CD4^+CD25^{hi}$ suppressor (▲) cells (2.5×10^3 cells/well) were stimulated with plate-bound anti-CD3 (0.1 and 0.5 μg/ml). $CD4^+CD25^-$ T cells were cocultured with $CD4^+CD25^{hi}$ T cells at a 1:1 ratio (○). T cell–proliferative responses are expressed as the mean ± SE of triplicate cultures. Culture supernatants were diluted and analyzed to determine the cytokine profile. In the series of experiments shown on the left, cytokine secretion was not measurable. In the subsequent series, IFNγ and IL-10 were detected; their values, representing the mean ± SE of triplicate cultures, are expressed as ng/ml.

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Proliferation Assay. $CD4^+CD25^-$ (responders) and $CD4^+CD25^{hi}$ (suppressors) T cells (2.5×10^3 cells/well) were stimulated with plate-bound anti-CD3 (clone UCHT1; BD Biosciences) used at three different concentrations (0.1, 0.5, and 2.5 μg/ml, coated in PBS for 2–4 h at 37°C), or soluble anti-CD3 (clone HIT3a at 5 μg/ml) together with soluble anti-CD28 (clone 28.2 at 5 μg/ml) in U-bottom 96-well plates. $CD4^+CD25^-$ T cells were also cocultured with decreasing numbers of $CD4^+CD25^{hi}$ T cells (responder/suppressor ratios: 1:1, 1:1/2, 1:1/4, and 1:1/8). All cells were cultured in a final volume of 200 μl in the presence of 10^4 T cell–depleted accessory cells/well. After 5 d of culture, 100 μl of supernatant was removed from each well and used for cytokine detection, and 1 μCi [^{3}H]thymidine (NEN Life Science Products) was added to each well. The cells were harvested after 16 h; cpm/well was determined by scintillation counting (PerkinElmer). Mixing experiments were performed in the presence of fixed accessory cells autologous to the $CD4^+CD25^-$ responder T cells.

Measurement of Cytokine Level. The supernatants removed from each well before [^{3}H]thymidine addition were diluted and analyzed to determine the cytokine profile by ELISA using Immulon 4 ELISA plates (Dynex Technologies). Antibody pairs and standards for IFNγ were purchased from Endogen. Abs for IL-13 and IL-10 were purchased from BD Biosciences. Avidin–peroxidase conjugate (Sigma-Aldrich) and tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories) were used to develop the assay. For IL-6 detection, the Human Inflammatory Cytometric Bead Array kit obtained from BD Biosciences was used.

Generation of $CD4^+CD25^{hi}$ Clones. PBMCs were stained with the aforementioned antibodies for flow cytometry. The $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ cells were single cell sorted into 96-well plates. The cells were stimulated (modification of method described by Levings et al.; reference 23) with PHA (Boehringer) at 0.05 μg/ml, autologous PBMCs (6,000 rad) were stimulated at 3×10^4 cells/well, JY cells (10,000 rad) were stimulated at 10^4 cells/well, and IL-2 (Teceleukin; National Cancer Institute) were stimulated at 100 U/ml. Half the medium was replaced at day 7, and every 3–4 d thereafter it was replaced with fresh medium containing 100 U/ml IL-2. The cultures were restimulated with 0.05 μg/ml PHA, a mixture of irradiated autologous ($1–3 \times 10^4$ cells/well) and allogeneic ($1–3 \times 10^4$ cells/well) PBMCs, and 100 U/ml IL-2. After 2–3 wk, growing clones were tested for regulatory activity as defined by suppression of cocultured anti-CD3–mediated T cell proliferation and the cloned cell dependence on exogenous IL-2 for proliferation. With these experiments, we further confirmed the data from Levings et al. showing the possibility of single cell cloning of $CD4^+CD25^{hi}$ regulatory cells. Clones were analyzed in cocultures containing 4×10^3 autologous $CD4^+CD25^-$ T cells or 4×10^3 PBMCs/well and 2×10^3 cloned cells/well. Stimulation was provided by the addition of magnetic beads with covalently attached anti-CD3 (UCHT1, bound to tosyl-activated magnetic beads at 1 μg/10^7 beads; Dynal Biotech Inc.). The beads were added to the cultures at 1.5×10^4 cells/well. ^{3}[H]Thymidine was added to the cultures after 4 d of stimulation. Percent suppression was determined as 1 − (cpm incorporated in the coculture)/cpm of responder population alone) × 100%.

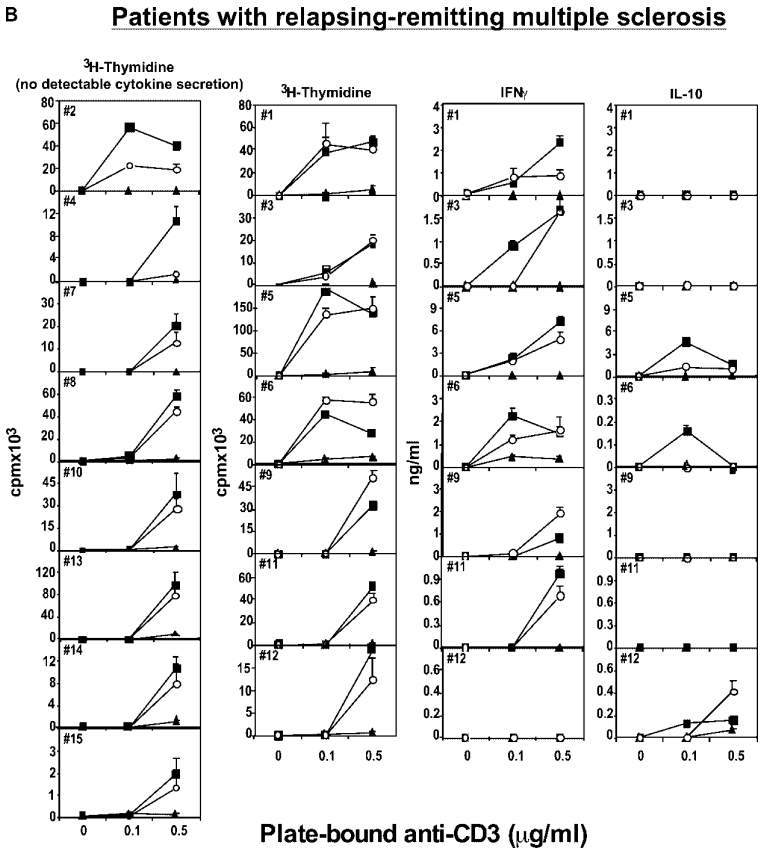

Figure 1 (continued)

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Statistical Analysis. The mean ± SE thymidine uptake and cytokine secretion of triplicate cultures was calculated for each experimental condition. The response of $CD4^+CD25^-$ T cells were normalized to 100 to calculate the percent suppression resulting from the addition of $CD4^+CD25^{hi}$ to the cultures. The Mann-Whitney test was used to evaluate possible differences in the $CD4^+CD25^{hi}$ function between patients with MS and healthy donors. The number of wells that produced growth from the cloning of cells derived from patients with MS and healthy controls was compared using hierarchical logistic regression analysis.

Results

$CD4^+CD25^{hi}$ T Cells Are Present with the Same Frequency in Healthy Donors and Patients with MS. We stained whole mononuclear cells from freshly drawn human blood with different combination of anti-CD4–CyChrome, anti-CD25–PE, and a cocktail of FITC-labeled anti-CD14, anti-CD32, and anti-CD116 antibodies. The cells were gated on lymphocytes via their forward and side scatter features, and all FITC-labeled cells were negatively selected during sorting. Human peripheral blood contains a heterogeneous population of $CD4^+CD25^+$ T cells that express either moderate levels of CD25 consisting of nonregulatory T cells or high levels of CD25 that exhibit regulatory function (24). As there are no other known cell surface markers able to identify regulatory T cells ex vivo, we used CD25 expression to discriminate regulatory T cells in humans. We analyzed the mean fluorescent intensity of the $CD25^+$ population in both patients with MS and control subjects and found no differences between the two groups. Similarly, no differences in the frequency of $CD4^+CD25^{hi}$ T cells were found between patients and healthy controls; ~10% of CD4 T cells express the α-chain of IL-2 receptor CD25, whereas only 1–2% of the CD4 T cells are $CD25^{hi}$ in both subject groups. The mean of frequency was 1.4% ± 0.3 in the healthy control group and 1.2% ± 0.2 in the MS patient group; no statistically significant differences were found between the two populations ($P = 0.21$).

$CD4^+CD25^{hi}$ Regulatory T Cells Display Impaired Function in Patients with MS. Because it was critical to examine the regulatory T cell function, we isolated highly pure $CD4^+CD25^{hi}$ regulatory and $CD4^+CD25^-$ responder cell populations by high speed flow cytometric sorting. $CD4^+CD25^-$ responder cells from both patients with MS and healthy individuals responded similarly in a dose-dependent fashion to varying concentration of plate-bound anti-CD3 mAb. $CD4^+CD25^{hi}$ T cells isolated from both groups were anergic to stimulation at all doses of plate-bound anti-CD3, indicating that $CD4^+CD25^{hi}$ T cells isolated from patients with MS do exhibit this regulatory property.

To quantitate their regulatory function, $CD4^+CD25^{hi}$ T cells were cocultured with autologous responder cells (2.5×10^3 cells/well) at different ratios (responder/suppressor ratios: 1:1, 1:1/2, 1:1/4, and 1:1/8). As reported previously in healthy individuals, $CD4^+CD25^{hi}$ T cells consistently suppressed proliferation at a 1:1 ratio (Figs. 1 A and 2). Increasing the ratio of responder/suppressor T cells resulted in less suppression (Fig. 2). In striking contrast, the regulatory $CD4^+CD25^{hi}$ T cells isolated from the circulation of patients with MS (while similar in frequency as compared with healthy controls) poorly inhibited responder $CD4^+CD25^-$ T cell proliferation (Figs. 1 B and 2). Because we have shown previously that increased strength of signal inhibits regulation (24), we stimulated cocultures of regulatory and responder T cells from patients with MS and healthy controls with a maximal concentration (2.5 μg/ml) of plate-bound anti-CD3 mAb. As predicted, the $CD4^+CD25^{hi}$ regulatory T cells no longer suppressed the proliferation of responder T cells (Fig. 2).

We also examined the production of cytokines in all the cultures and the ability to inhibit their secretion by $CD4^+CD25^{hi}$ T cells cocultured with $CD4^+CD25^-$ responder cells. The secretion of the Th1 cytokine IFNγ, as would result from the activation of destructive, autoreactive T cells, was suppressed in $CD4^+CD25^{hi}$ T cell cocultures from healthy controls, but not in cocultures derived from patients with MS (Fig. 1, A and B). Because there was no detectable secretion of IL-13 at the cell numbers used in these experiments, those data are unpublished. IL-10 was variably secreted, predominantly by the $CD4^+CD25^-$ T cells. The secretion of IL-10 was often reduced upon coculture with $CD4^+CD25^{hi}$ T cells, excluding a potential role of this cytokine in mediating this regulatory suppressor function (Fig. 1, A and B). In this ex vivo model of suppression, blocking IL-

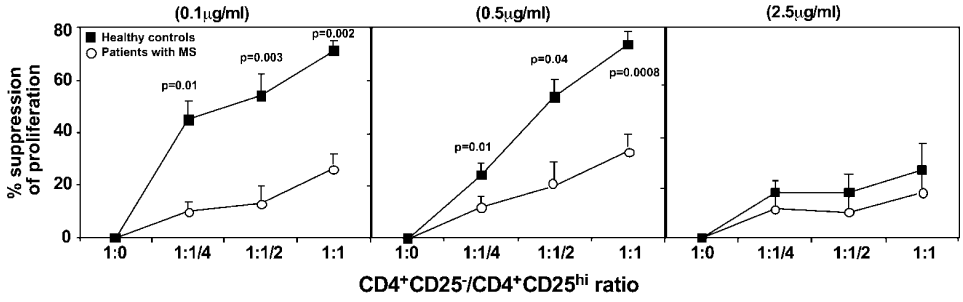

Figure 2. Summary of $CD4^+CD25^{hi}$ T cell regulatory function that is altered in patients with MS. The mean percent inhibition of the proliferative response by $CD4^+CD25^{hi}$ cells derived from 15 MS patients (○) and 21 healthy controls (■) was calculated. $CD4^+CD25^-$ and $CD4^+CD25^{hi}$ populations were stimulated with plate-bound anti-CD3 mAb, alone or cocultured at varying ratios. The proliferative response was inhibited upon addition of $CD4^+CD25^{hi}$ cells to the $CD4^+CD25^-$ responder cells at a 1:1 ratio in normal controls. Regulatory T cells from patients with MS exhibited significantly less suppressor activity. Decreasing the number of $CD4^+CD25^{hi}$ T cells (responder/suppressor ratios: 1:1/2 and 1:1/4) resulted in less suppression in all the conditions examined.

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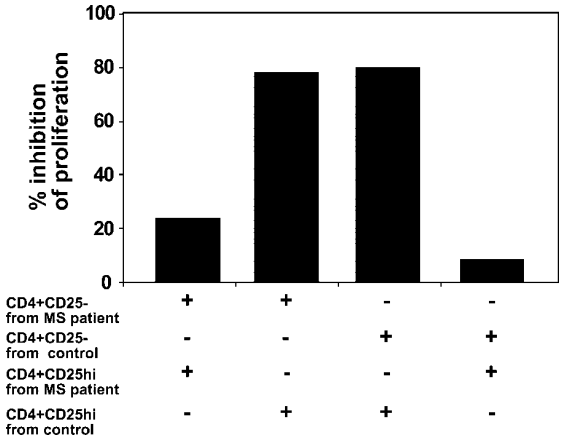

Figure 3. $CD4^+CD25^{hi}$ T cells from patients with MS do not inhibit proliferation of responder T cells isolated from either the autologous individual or healthy donors. $CD4^+CD25^-$ responder T cells and $CD4^+CD25^{hi}$ cells from MS patients and normal controls were stimulated with plate-bound anti-CD3 at 0.5 μg/ml. 2.5×10^3 cells/well responder T cells from MS patients were cocultured with the same number of autologous $CD4^+CD25^{hi}$ regulatory T cells (1st bar) or with regulatory cells isolated from healthy donors (2nd bar). Conversely, responder T cells from healthy donors were cocultured either with $CD4^+CD25^{hi}$ cells derived from the same subject (3rd bar), or with regulatory cells isolated from patients with MS (4th bar).

10 or TGFβ does not result in loss of suppressor function by $CD4^+CD25^+$ regulatory T cells (unpublished data).

Recent studies have shown that the presence of IL-6 can contribute to the loss of $CD4^+CD25^+$ suppression (25). We measured the levels of IL-6 secretion in the cultures of five healthy individuals and four patients with MS to investigate its potential role in the lack of suppression observed with patient-derived cells. We found that IL-6 levels did not correlate with the presence or absence of $CD4^+CD25^{hi}$ T cell–mediated suppression.

It was also important to examine whether the loss of regulatory function was due to a decrease in $CD4^+CD25^{hi}$ T cell function or an increase in the resistance of activated $CD4^+CD25^-$ responder T cells to inhibition. Thus, we performed mixing experiments in which patient and control regulatory $CD4^+CD25^{hi}$ T cells were cocultured with the autologous and the converse target cells isolated from either healthy subjects or patients with MS. Regulatory T cells from patients with MS could not suppress the proliferative response of target responder T cells from either patients or healthy controls (suppression ≤23%). In contrast, in the reciprocal experiments, regulatory $CD4^+CD25^{hi}$ T cells from healthy controls suppressed the proliferative response of target $CD4^+CD25^-$ T cells derived from both controls and patients with MS (suppression ≥78%; Fig. 3). These data indicate that the primary regulatory defect is in the function of $CD4^+CD25^{hi}$ T cells isolated from the circulation of patients with MS.

CD62L Expression on $CD4^+CD25^{hi}$ Regulatory T Cells. Although there were no differences in the frequency of $CD4^+CD25^{hi}$ T cells or in their proliferation or cytokine secretion in response to different stimuli between healthy subjects and patients with MS, it was important to determine whether an increase in the frequency of activated $CD4^+$ T cells in the circulation of patients with MS may be diluting the regulatory $CD4^+CD25^{hi}$ T cells. Therefore, we further restricted the isolation of T regulatory cells by sorting only those $CD4^+CD25^{hi}$ T cells that expressed CD62L because L-selectin expression is down-regulated upon activation. We isolated $CD4^+CD25^{hi}CD62L^+$ and total $CD4^+CD25^{hi}$ regulatory T cells from healthy subjects and patients with MS and found that, whereas in the healthy controls both populations were able to suppress the proliferative response to anti-CD3 stimulation, neither population of regulatory cells isolated from patients with MS was able to inhibit the proliferation of the $CD4^+CD25^-$ responder population (Fig. 4). The fact that the $CD4^+CD25^{hi}CD62L^+$ T cells isolated from patients are depleted of the potentially activated $CD62L^-$ T cells yet still unable to suppress indicates that the impaired regulatory T cell function in patients with MS is not due to contamination by activated T cells.

In Vivo Immunization Did Not Alter $CD4^+CD25^{hi}$ Regulatory T Cell Function. To further rule out the possibility that impaired suppressive function observed in patients with MS was due to an increase in the frequency of activated $CD4^+$ T cells in the circulation or due to an altered activation state of the responder cells, we examined the

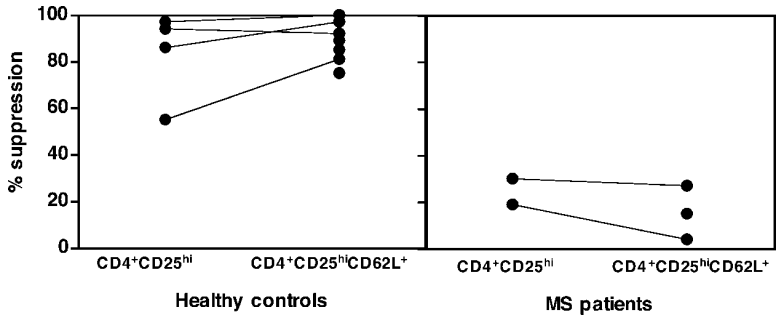

Figure 4. The $CD62L^+$ subset of $CD4^+CD25^{hi}$ regulatory T cells exhibits decreased suppressive function in patients with MS. $CD4^+CD25^{hi}CD62L^+$ T cells were isolated from seven healthy individuals (left) and three patients with MS (right). Each symbol is representative of a different individual. Total $CD4^+CD25^{hi}$ T cells were also isolated from four out of seven healthy subjects and two out of three patients. The percent suppression of coculture proliferation in response to plate-bound anti-CD3 was calculated in each individual. The $CD4^+CD25^{hi}$ regulatory T cells derived from healthy controls were able to induce strong inhibition of the proliferative response (55–97% suppression) as shown previously. The $CD62L^+$ subset of $CD4^+CD25^{hi}$ T cells derived from the same individuals exhibit enhanced suppressive capacity. In contrast, the regulatory cells derived from patients, although depleted of $CD62L^-$ activated T cells, show decreased inhibitory function (4–29% suppression) as compared with the suppression observed in healthy individuals.

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