Dysregulated T cell expression of TIM3 in multiple sclerosis

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T cell immunoglobulin- and mucin domain–containing molecule (TIM)3 is a T helper cell (Th)1–associated cell surface molecule that regulates Th1 responses and promotes tolerance in mice, but its expression and function in human T cells is unknown. We generated 104 T cell clones from the cerebrospinal fluid (CSF) of six patients with multiple sclerosis (MS) ($n = 72$) and four control subjects ($n = 32$) and assessed their cytokine profiles and expression levels of TIM3 and related molecules. MS CSF clones secreted higher amounts of interferon (IFN)-γ than did those from control subjects, but paradoxically expressed lower levels of TIM3 and T-bet. Interleukin 12–mediated polarization of CSF clones induced substantially higher amounts of IFN-γ secretion but lower levels of TIM3 in MS clones relative to control clones, demonstrating that TIM3 expression is dysregulated in MS CSF clones. Reduced levels of TIM3 on MS CSF clones correlated with resistance to tolerance induced by costimulatory blockade. Finally, reduction of TIM3 on ex vivo CD4$^+$ T cells using small interfering (si)RNA enhanced proliferation and IFN-γ secretion, directly demonstrating that TIM3 expression on human T cells regulates proliferation and IFN-γ secretion. Failure to up-regulate T cell expression of TIM3 in inflammatory sites may represent a novel, intrinsic defect that contributes to the pathogenesis of MS and other human autoimmune diseases.

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Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) believed to be initiated and mediated by autoreactive T cells directed against myelin antigens (1). It has been hypothesized that Th1 cells secreting proinflammatory cytokines including IFN-γ and TNF-α contribute to the pathogenesis of MS, whereas Th2 cells secreting IL-4, IL-5, and IL-13 ameliorate the inflammatory process and disease (2). Transcription factors and cell surface molecules have been identified that are tightly associated with Th1 or Th2 cells (3, 4). Although it does not promote Th1 differentiation, T cell immunoglobulin- and mucin domain–containing molecule (TIM)3 is selectively expressed on fully differentiated Th1, but not Th2 cells (5). More recently, engagement of TIM3 with TIM3 ligand has been shown to regulate both the function of Th1 cells and the ability to induce tolerance, as blockade of the TIM3 pathway accelerates diabetes in the nonobese

diabetes (NOD) mice model of diabetes and prevents the acquisition of transplantation tolerance induced by costimulatory blockade (6). Furthermore, TIM3-deficient mice are refractory to induction of high dose tolerance in experimental autoimmune encephalomyelitis (EAE) (7).

Here, we explored the mechanism for increased IFN-γ secretion by CD4$^+$ T cells in the cerebrospinal fluid (CSF) of patients with MS, postulating that alterations in the TIM3 pathway may underlie the enhanced IFN-γ secretion observed in patients with the disease. We generated 104 independent T cell clones from the CSF of MS patients and control subjects; cytokine secretion was measured and quantitative RT-PCR was used to analyze the specificity and stability of transcription factors that dictate Th1 and Th2 programs. Paradoxically, MS CSF clones express higher levels of IFN-γ and they express lower levels of messenger RNA (mRNA) for both T-bet and TIM3. In vitro polarization experiments with CSF clones from MS and control subjects demonstrate robust
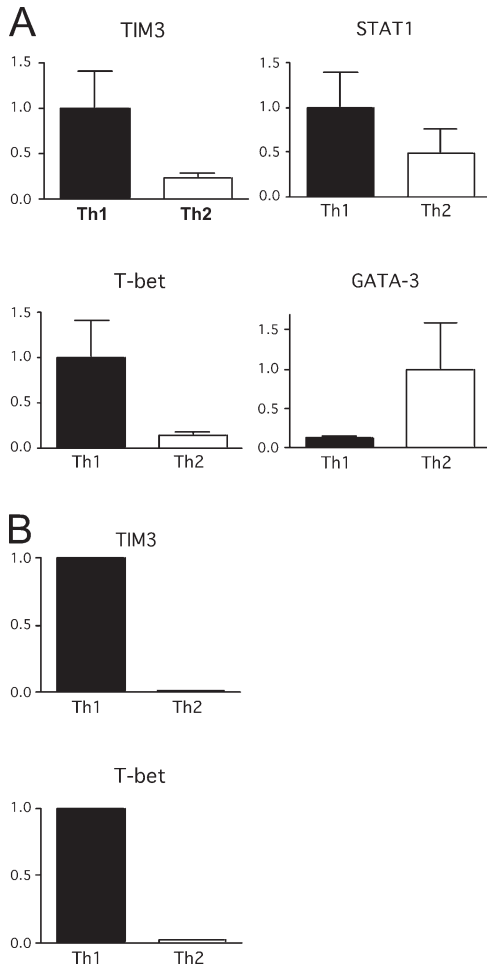
Figure 1.   Differential expression of T-bet, TIM3, and GATA-3 among Th1 and Th2 T cell clones. Expression levels of TIM3, T-bet, STAT1, and GATA-3 were examined by quantitative RT-PCR. Relative expressions of each transcript have been normalized to a single clone expressing the highest level of a transcript. (A) CD4+ T cell clones from the CSF of control subjects that secreted >100 pg/ml of IFN-γ and <100 pg/ml of IL-13 were defined as Th1 ($n = 9$), whereas those secreting >100 pg/ml of IL-13 and <100 pg/ml of IFN-γ were defined as Th2 ($n = 6$). Error bars represent the standard deviation of mRNA levels among the clones. (B) Highly polarized Th1 and Th2 clones were generated from the peripheral blood of a healthy normal individual. Levels of TIM3 and T-bet transcripts are depicted from representative Th1 and Th2 clones as described in A.

enhancement of IFN-γ secretion, but a relative inability to up-regulate TIM3 in MS CSF clones. Using small interfering (si)RNA to reduce TIM3 expression on CD4+ T cells and IFN-γ secretion when TIM3 expression was inhibited, providing a mechanistic link between our observations of lower TIM3 levels and elevated IFN-γ secretion among MS CSF clones. These results are the first human data to demonstrate that TIM3 expression regulates T cell secretion of IFN-γ. Moreover, these data suggest that TIM3 may critically regulate self-reactive Th1 cells and maintenance of tolerance in human autoimmune disease.
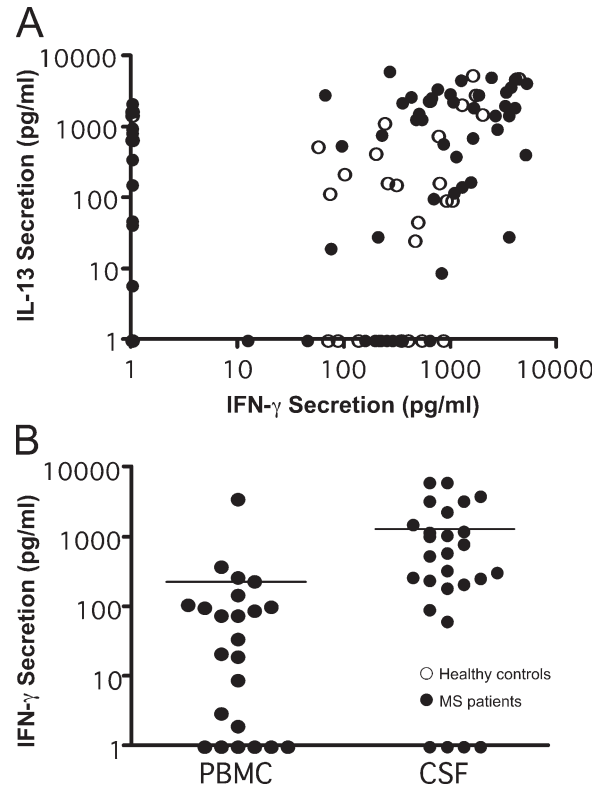

Figure 2.   Cytokine profiles of CSF clones from MS and control subjects. (A) CD4+ T cell clones derived from the CSF of MS patients ($n = 72$) or control subjects ($n = 32$) were expanded and stimulated with 2.0 μg/ml of plate-bound anti-CD3 monoclonal antibody for 2 d. Supernatants were collected and IFN-γ and IL-13 were measured by ELISA. Levels of cytokine secretion in the absence of stimulation have been subtracted from all reported values. T cells from patients with MS secreted significantly higher amounts of IFN-γ ($P = 0.033$) and comparable amounts of IL-13 ($P = 0.147$) relative to those from control subjects. (B) T cell clones derived from both the peripheral blood (PBMC) ($n = 23$) and CSF ($n = 27$) of three MS patients were expanded and their cytokine profiles were characterized as described in A. CSF clones secreted significantly greater amounts of IFN-γ than did those from peripheral blood ($P = 0.009$).

RESULTS AND DISCUSSION

Stability and specificity of markers of human Th1 cells

We generated a total of 104 independent CD4+ T cell clones from the CSF of patients with MS ($n = 6$) and control subjects ($n = 4$) and examined their cytokine profiles using ELISA after stimulation with anti-CD3 monoclonal antibody. We selected several Th1 and Th2 clones from healthy subjects and examined mRNA expression levels of Th1- and Th2-associated molecules using quantitative RT-PCR. Levels of TIM3 and T-bet in T cell clones isolated from control subjects were expressed at substantially higher levels in Th1 clones, whereas GATA-3 was substantially elevated in Th2 cells (Fig. 1 A). Levels of STAT1 and STAT4 tended to be higher on Th1 clones, but the differences were not statistically substantial (Fig. 1 A and not depicted). We confirmed that TIM3 and T-bet are differentially expressed on representative Th1 or Th2 clones differentiated from PBMCs of healthy subjects (Fig. 1 B).

A

B

○ Healthy controls
● MS patients
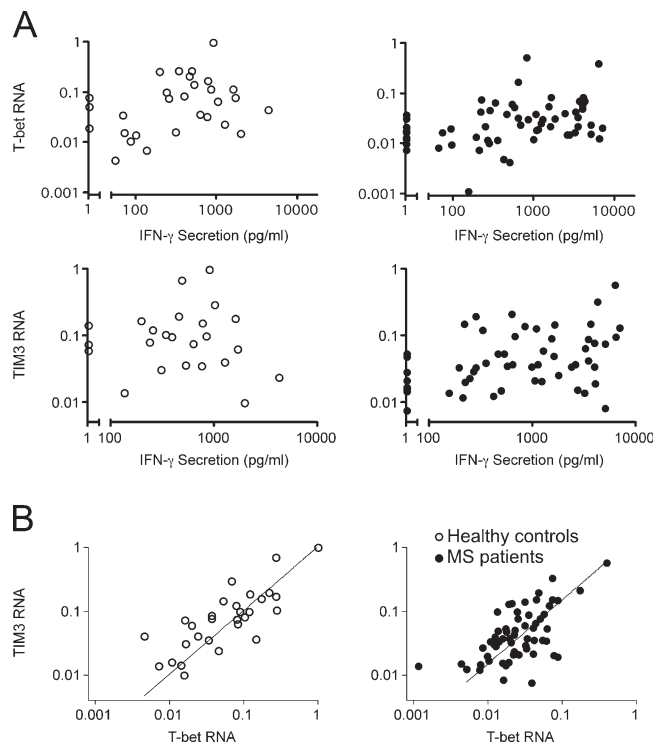
Figure 3.   Altered levels of TIM3, T–bet, and STAT1 among T cell clones from MS patients. (A) Expression levels of T–bet and TIM3 were examined by quantitative RT-PCR in the panel of CSF-derived T cell clones described in Fig. 2 A. Expression levels of each transcript have been normalized to a single clone expressing the highest level of a transcript. Clones from MS patients expressed significantly lower levels of T–bet (P = 0.014) and TIM3 (P = 0.029). There were no substantial differences in levels of STAT4, TIM1, and GATA-3 (not depicted). Note the greater percentage of clones in patients compared with control subjects that secrete high amounts of IFN-γ, yet express relatively low levels of TIM3 (bottom right regions). (B) Despite differences in levels of T–bet and TIM-3 expression between healthy subjects and MS patients, transcript levels were highly correlated among all T cell clones (R = 0.9651 and 0.6609, respectively).

Although a subset of CSF-derived T cell clones had highly polarized Th1 or Th2 phenotypes, the majority of clones derived from both MS patients and control subjects had a Th0-like cytokine profile, secreting both IFN-γ and IL-13, though clones from MS patients secreted higher amounts of IFN-γ (P < 0.05) (Fig. 2 A). Furthermore, when comparing clones generated from the peripheral blood versus CSF of the same patients with MS, CSF clones secreted significantly higher levels of IFN-γ (P = 0.009) (Fig. 2 B). These data are consistent with previous observations at the mRNA level that MS CSF clones more frequently secrete IFN-γ compared with control CSF clones than do clones isolated from the peripheral blood of the same patients (8, 9), and suggest that Th1 polarization of human T cells may be more apparent within the target organ.

Reduced TIM3 expression among MS T cell clones
Despite the fact that clones from patients with MS collectively secreted higher levels of IFN-γ, we found that they

expressed on average lower levels of transcripts for T–bet and TIM3 than did clones from healthy subjects (P < 0.05 for both molecules, Fig. 3 A). Though levels of T–bet and TIM3 were reduced in clones from patients with MS, there was nevertheless a strong correlation between transcript levels of T–bet and TIM3 in both MS patients and healthy subjects (Fig. 3 B). This observation argues against defects in transcriptional regulation of T–bet or TIM3 that are independent of one another, and instead suggests that these two molecules are coordinately regulated. Because T–bet is a transcription factor involved in the transcription of IFN-γ and other Th1 genes (10), this raises the issue of whether T–bet can directly transactivate or indirectly regulate the expression of TIM3.

Impaired induction of TIM3 in MS CSF clones
To directly demonstrate a defect in TIM3 regulation in the MS CSF clones, we further polarized clones by repeated stimulation in the presence of recombinant IL-12 and anti–IL-4 monoclonal antibody and measured levels of IFN-γ secretion and TIM3 expression. All clones secreted comparable, low amounts of IFN-γ (61.7 ± 22 and 58 ± 28 pg/ml, respectively, for control and MS CSF clones) and after polarization, all secreted substantially higher levels of IFN-γ. However, MS CSF T cell clones secreted significantly higher levels of IFN-γ than did those from control subjects, but expressed lower levels of TIM3 mRNA (P = 0.01) (Fig. 4 A). These data directly demonstrate that there is a defect in induction of TIM3 among MS CSF T cell clones.

Given previous reports that TIM3 regulates tolerance (6, 7), we hypothesized that the relative inability to up-regulate TIM3 on MS CSF clones would render them resistant to tolerance mediated by costimulatory blockade. MS and control CSF clones were stimulated with anti-CD3 monoclonal antibody in the presence or absence of CTLA-4 Ig to induce tolerance. After 7 d of culture, cells were collected and restimulated and production of IFN-γ was measured. Consistent with our hypothesis, MS CSF clones expressing lower levels of TIM3 than control clones were resistant to tolerance (P = 0.01) (Fig. 4 B).

IFN-γ increases levels of STAT1 mRNA through transcriptional regulation (11) and STAT1 can directly activate T–bet in T cells (12), suggesting that levels of IFN-γ should correlate with STAT1 signaling and in turn influence levels of T–bet expression. We measured levels of STAT1 transcripts among CSF clones from patients with MS and healthy controls and found that MS clones expressed significantly higher levels (P = 0.02; unpublished data), consistent with enhanced IFN-γ secretion by these clones. To determine if IFN-γ signaling through STAT1 was intact in MS T cell clones, we exposed clones from MS patients and control subjects to recombinant IFN-γ and measured levels of total versus activated, tyrosine-phosphorylated forms of STAT1 protein. Consistent with mRNA levels of STAT1, resting levels of total STAT1 protein were higher in T cell clones from patients (Fig. 4 C). Exposure to increasing doses of IFN-γ increased levels of phosphorylated STAT1 in MS clones, but remained

A

B

C
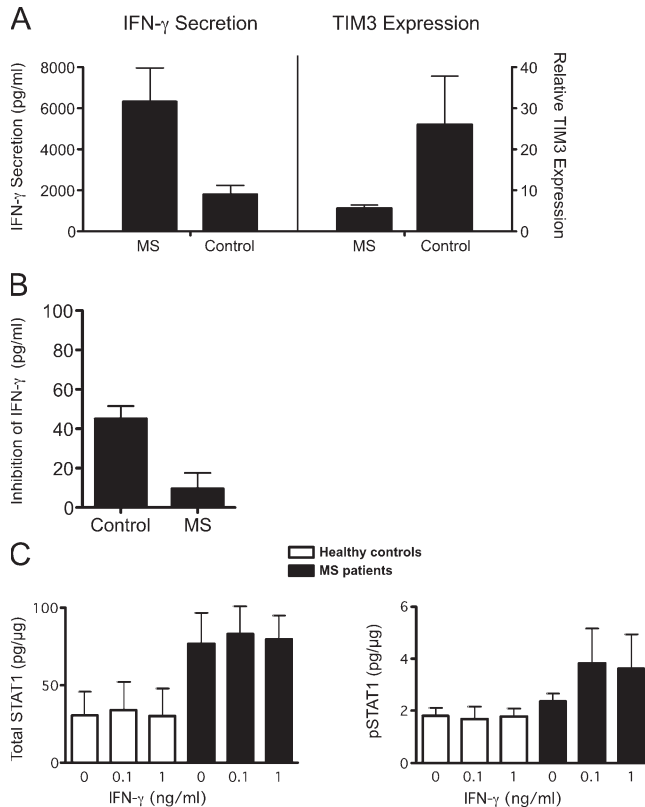
Figure 4. Dysregulated TIM3 induction and function among MS CSF T cell clones. (A) Relative inability to induce TIM3 expression on MS CSF clones. CSF T cell clones from MS patients (*n* = 5) and healthy subjects (*n* = 6) that secreted comparable levels of IFN-γ (61.7 ± 22 and 58 ± 28 pg/ml, respectively, for control and MS CSF clones) were subjected to two rounds of Th1 polarization, at which point TIM3 expression and IFN-γ secretion after stimulation were determined. CSF T cells from patients with MS secreted significantly higher amounts of IFN-γ but not TIM3 (P = 0.01), demonstrating that they are impaired in their ability to up-regulate TIM3. (B) Reduced tolerance induction in MS CSF clones. Control and MS CSF clones were stimulated with plate-bound anti-CD3 mAb in the presence/absence of CTLA-4 Ig to induce tolerance. After 7 d, cells were collected and restimulated with anti-CD3 mAb, and supernatants were collected after 48 h for measurement of IFN-γ. On average, MS CSF clones expressed 3.3-fold less TIM3 than did control CSF clones and were resistant to tolerance induction (P = 0.01). (C) IFN-γ–mediated STAT1 signaling is functional in MS CSF clones. CSF T cell clones from MS patients and control subjects were cultured in the presence or absence of 0.1 or 1 ng/ml of recombinant IFN-γ for 3 h, after which time levels of total STAT1 protein and active STAT1 protein were determined by ELISA (R&D Systems). Error bars represent differences in levels of total or active STAT1 protein among three clones examined from MS patients and from healthy subjects.

unchanged in clones from control subjects, perhaps the result of an increased sensitivity to IFN-γ signaling in MS clones as a result of their higher overall levels of STAT1 protein.

siRNA–mediated reduction of TIM3 enhances T cell proliferation and IFN-γ secretion

To provide a mechanistic link between dysregulated TIM3 expression and IFN-γ secretion in CSF clones from MS

patients, we isolated CD4^{+} T cells from the peripheral blood of healthy subjects and used siRNA to reduce expression of TIM3 on T cells. The T cells were subsequently stimulated with plate-bound anti-CD3/CD28 monoclonal antibodies and their proliferative capacity and ability to secrete IFN-γ were examined. Inhibition of TIM3 expression on CD4^{+} T cells substantially enhanced proliferation and IFN-γ secretion (Fig. 5). We were unable to perform the reciprocal experiment and restore TIM3 expression in the MS CSF clones because of an inability to transduce the clones with a lentiviral vector. These data using ex vivo CD4^{+} T cells validate the physiological relevance of observations made using our large panel of CSF-derived T cell clones. Moreover, they directly demonstrate that down-modulation of TIM3 expression in human CD4^{+} T cells enhances both IFN-γ secretion and the potential for clonal expansion.

Although CSF T cell clones from patients with MS secreted higher amounts of IFN-γ than did clones from control subjects, they expressed lower levels of mRNA for both T-bet and TIM3. These observations were confirmed experimentally in vitro. That is, when polarized further under Th1 conditions that induce T-bet and TIM3 expression, clones from control subjects up-regulated TIM3 to much greater levels than did T cell clones from patients with MS, despite the fact that clones from the MS patients secreted higher levels of IFN-γ. Thus, there is dysregulation of TIM3 expression among CSF clones from MS patients.

Our data may initially appear paradoxical in that levels of T-bet, the master regulator of Th1 cells, are expressed at lower levels in MS CSF T cell clones that secrete substantially higher levels of IFN-γ, relative to CSF clones from healthy subjects. T-bet expression is low but not absent in the MS CSF clones; we postulate that the levels of T-bet in the MS clones were sufficient to allow initial commitment of the T cells toward IFN-γ production and that elevated IL-12/STAT4 signaling in patients with MS (13, 14) compensated for reduced T-bet expression to promote elevated IFN-γ secretion in these clones. Consistent with this hypothesis, culture of MS CSF clones in the presence of IL-12 dramatically enhances IFN-γ secretion (Fig. 4 A). Of note is the recent report that mice deficient for STAT1 are highly susceptible to EAE, despite reduced levels of IFN-γ secretion and reduced levels of T-bet (15). Thus, reduced levels of T-bet do not necessarily predict reduced incidence of EAE or MS pathogenicity. It will be important to determine if there are additional abnormalities in regulation of proinflammatory cytokine secretion from the CSF T clones that we have isolated from MS patients.

In a previous study, it was found that human Th1 lines expressed higher levels of TIM-3 mRNA, whereas Th2 lines expressed higher levels of TIM-1 (16). Moreover, analysis of CSF mononuclear cells obtained from MS patients for mRNA levels of TIM1 and TIM3 indicated that higher mRNA expression of TIM-1 was associated with clinical remissions and low expression of IFN-γ. However, given that TIM3 is expressed by CD4^{+}, CD8^{+}, and CD11b^{+}

A

B

C
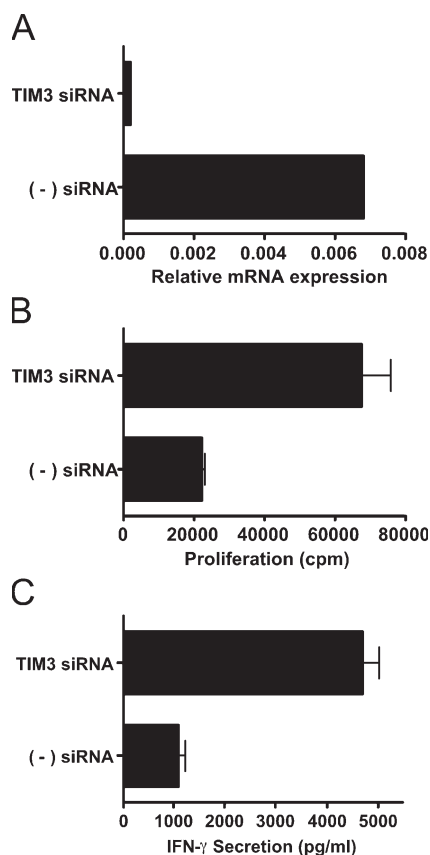
Figure 5.   TIM3 siRNA enhances T cell proliferation and IFN–γ secretion. (A) Ex vivo CD4$^+$ T cells were transfected with TIM3 or negative control oligonucleotides and TIM3 mRNA levels were determined. After transfection, cells were collected and 2.5 × 10^4 cells/well were stimulated in triplicate with plate-bound anti-CD3/CD28 monoclonal antibodies. Proliferation measured using tritiated thymidine incorporation (B) and IFN-γ secretion (C) were measured after 48 h. Results are representative of three independent experiments using blood from three different donors. Similar observations were seen using a second TIM3 siRNA oligonucleotide and when transfecting polarized Th1 cell lines. Thus, RNAi inhibition of TIM3 expression induces a CD4 phenotype of the CSF-derived MS T cells. Error bars represent the standard deviation of triplicate wells in B and C.

macrophages (5), all of which can be found in MS CSF fluid and contribute to the pathogenesis of MS, a major limitation of the previous study was analysis of TIM3 and cytokine levels using unfractionated CSF mononuclear cells. It was unclear which populations of cells expressed TIM3 and the detected cytokines, and relative proportions of these populations likely differed among patients. The present study has allowed us to demonstrate that there is dysregulation of TIM3 expression and IFN-γ secretion specifically among CD4$^+$ T cells from the CSF of MS patients.

TIM3 expression and pathophysiology of MS
In mice, the interaction of TIM3 with TIM3 ligand appears to regulate both the function of Th1 cells and the ability to induce tolerance (6, 7). In addition, TIM3-deficient mice are

refractory to the induction of high dose tolerance (7). Finally, galectin-9 has recently been identified as a ligand of TIM3 and has been shown to suppress IFN-γ secretion (17). Our data suggest that human TIM3 may similarly play a role in the regulation of Th1 cells and maintenance of tolerance in the context of MS. Specifically, we demonstrate that reduction of TIM3 expression on human CD4$^+$ T cells enhances both proliferation and IFN-γ secretion after T cell stimulation and that CSF clones from MS patients express lower levels of TIM3 than do those from control subjects, yet secrete higher levels of IFN-γ. Moreover, T cell clones derived from the CSF of patients with MS that express lower levels of TIM3 than control clones are resistant to tolerance induction. In conclusion, our data indicate that reduced TIM3 expression may allow uninhibited expansion of IFN-γ–secreting cells in the target organ. Indeed, failure to up-regulate T cell expression of TIM3 in inflammatory sites may represent a novel, intrinsic defect that may contribute to the pathogenesis of MS and other human autoimmune diseases.

MATERIALS AND METHODS
Subjects. CSF samples were obtained from four control subjects and six MS patients with Kurtzke Expanded Disability Status Scale scores between 0 and 2. Cerebral spinal fluid was drawn from control subjects to rule out encephalitis and patients were uniformly negative for any CNS infection or autoimmune disease. All patients were female and their mean age was 42 yr old (range 29–59 yr). No patients had received immunomodulatory drugs or corticosteroids within 3 mo before the time at which samples were collected. All samples were obtained in compliance with the Brigham and Women's Hospital institutional review board protocol.

Generation of T cell clones. T cell clones were derived from CSF samples by limiting dilution cloning and mitogenic expansion as we have described previously (18). Polarized Th1 and Th2 cells were generated using PBMCs from healthy subjects as we have described previously (19). Some CSF clones from MS patients and control subjects were further polarized using the same Th1 polarizing conditions.

Proliferation assay and ELISA measurements. T cell clones were stimulated with 2 μg/ml of plate-bound anti-CD3 (clone UCHT1; BD Biosciences) and, after 48 h, supernatants were collected and IFN-γ and IL-13 cytokine production was determined by ELISA; proliferation was measured using a liquid scintillation counter (PerkinElmer) as we have described previously (19).

Anergy assay. MS ($n = 3$) and control ($n = 4$) CSF clones that on average differed in TIM3 expression 3.3-fold were stimulated with plate-bound anti-CD3 mAb in the presence of CTLA-4 Ig or control Ig and cultured for 7 d, at which point T cells were collected, washed, and restimulated with plate-bound anti-CD3 mAb. Supernatants were collected after 48 h for measurement of IFN-γ by ELISA.

TIM3 siRNA. CD4$^+$ T cells were isolated using the CD4$^+$ T cell isolation Kit II (Miltenyi Biotec) from PBMCs. Predesigned siRNA for human TIM3 and a negative control siRNA were obtained from Ambion. Purified CD4$^+$ T cells (2.5 × 10^6) were transfected with either negative control siRNA or TIM3 siRNA (30 pmol siRNA/transfection) by electroporation, using the Nucleofactor Device (Amaxa Inc.). Transfected cells were incubated at 37°C for 30 min, followed by addition of 20 U/ml of human IL-2 and transfer of the cells to a 96-well plate coated with anti-CD3/CD28 monoclonal antibodies (3 μg/ml and 1 μg/ml, respectively; BD Biosciences). Plates were incubated at 37°C for 48 h, at which time IFN-γ secretion and proliferation

were determined. Cells from replicate wells were collected for RNA extraction using TRIzol reagent to assess the extent of down-modulation of TIM3 (Invitrogen). Reverse transcription was performed using a First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using ABI PRISM 7700 Detection System (PerkinElmer).

Quantitative RT–PCR. T cell clones were lysed and total RNA was extracted using Qiagen Rneasy Mini Kits (QIAGEN). Samples were digested with DNase 2 times (QIAGEN RNase-free DNase set and Invitrogen Deoxyribonuclease I Amplification Grade). Reverse transcription was performed using TaqMan Reverse Transcription Reagent (Applied Biosystems). The sequences of primers and probe for TIM3 have been described previously (16), and those for the housekeeping gene GAPDH were obtained commercially (Applied Biosystems). The sequences of primers and probes of T-bet, GATA3, STAT1, and STAT4 are available upon request. The amount of target RNA in each sample was normalized by first obtaining the ΔCT value for each target (the difference in cycle threshold value of the target RNA and GAPDH). The relative expression of each target among the clones was normalized to an arbitrary maximum value of 1.0 represented by the sample with the lowest ΔCT. All samples were transcribed with and without reverse transcriptase to exclude the possibility of genomic contamination.

Statistics. Unpaired Student's *t* test was used for all statistical comparisons using Prism 4.0 software (GraphPad Software).

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