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Lack of TIM-3 Immunoregulation in Multiple Sclerosis

Li Yang, David E. Anderson, Juhi Kuchroo, and David A. Hafler

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS white matter associated with T cell infiltrates and alterations of immune functions that can be measured in the peripheral immune system. TIM-3 has been identified as a central regulator of IFN-γ-secreting type 1 Th (Th1) cells and immune tolerance. In this study, using a newly generated mAb against human TIM-3, we examined TIM-3 function on ex vivo CD4 \(^+\) T cells isolated from the circulation of healthy subjects and patients with MS. Blocking TIM-3 during T cell stimulation significantly enhanced IFN-γ secretion in control subjects but had no effect in untreated patients with MS, demonstrating a defect in TIM-3 immunoregulation. Treatment with glatiramer acetate or IFN-β reversed this functional defect. Reduced levels and altered kinetics of T cell TIM-3 expression, which was restored in treated patients, is one mechanism that can explain the loss of TIM-3 regulation of T cell function in untreated patients with MS. These data provide functional, mechanistic data for dysregulated TIM-3 immunoregulation in a human autoimmune disease and suggest that approved therapies for the treatment of MS may function in part by restoring TIM-3 immunoregulation of T cell function. The Journal of Immunology, 2008, 180: 4409–4414.

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS myelin characterized by focal T cell and macrophage infiltrates that lead to demyelination and loss of neurologic function (1). Acute MS plaques are characterized by the presence of activated CD4 \(^+\) and CD8 \(^+\) T cells as well as activated macrophages/microglia (2–4). The presence of these cells, in addition to inflammatory cytokines including IFN-γ, TNF-α, IL-17, and IL-12 (5–9), has led to the hypothesis that MS is a Th1-type cell-mediated autoimmune disease, with Th17 cells also contributing to the pathogenesis of the disease. IL-12 and IFN-γ promote the differentiation of Thp cells into Th1-type cells both in vitro and in vivo (10–12). In contrast, IL-4 is required for maturation of naïve Thp cells into Th2 cells (11, 13). Recent data demonstrate that TGF-β and IL-6 are responsible for the differentiation of naïve murine Thp cells into Th17 cells, with IL-23 critical for expansion of these cells (14–16). The cytokine(s) responsible for Th17 differentiation of naïve human Thp cells remain unclear, although IL-23 can induce IL-17 from memory human CD4 \(^+\) T cells (17, 18). Although it may be incorrect to assume that populations of human T cells will simply fall into categories of Th1, Th2, or Th17 cytokine secretion patterns, it does appear likely that the polarized secretion of cytokines by T cells under chronic inflammatory conditions may also occur in humans.

Tim-3 is a transmembrane protein expressed on differentiated Th1 or Th17 cells but not on naïve T cells or B cells (19). Engagement of murine (m) Tim-3 with its ligand galectin-9 serves to down-regulate Th1 responses (20). Moreover, gene disruption of mTim-3 prevents induction of tolerance. These data indicate that interaction of TIM-3 with TIM-3 ligand may serve to inhibit effector Th1 cells during a normal immune response and may be crucial for the induction of peripheral tolerance (20–22). We have recently demonstrated that T cell clones isolated from the cerebrospinal fluid of patients with MS secrete higher levels of IFN-γ than do control cerebrospinal fluid clones, yet express lower levels of human TIM-3, which provides supportive human data that human TIM-3 may regulate self-reactive Th1 cells in human autoimmune diseases (23). In the present study, we have assessed functionally TIM-3 immunoregulation of ex vivo CD4 \(^+\) T cell responses in untreated and treated patients with MS, relative to control subjects. We demonstrate that during T cell stimulation, the presence of a novel blocking anti-TIM-3 mAb significantly enhanced IFN-γ secretion in control subjects but had no effect in untreated patients with MS, demonstrating a defect in TIM-3 immunoregulation. However, treatment with glatiramer acetate or IFN-β reversed this functional defect. Reduced levels and altered kinetics of T cell TIM-3 expression, which was restored in treated patients, is one mechanism that can explain the loss of TIM-3 regulation of T cell function in untreated patients with MS. Collectively, these data demonstrate dysregulated TIM-3 function in a human autoimmune disease and suggest that immunoregulation by this pathway should be explored in other inflammatory human autoimmune diseases.

Materials and Methods

Subjects

Peripheral blood was obtained after informed consent from healthy subjects (n = 58) and MS patients (n = 95, mean age, 43.72 years; mean expanded disability status scale (EDSS) score, 1.53). All patients were seen at the Partners MS Center at Brigham and Women’s Hospital (Boston, MA). MS patients consisted of a group of untreated relapsing-remitting MS patients (n = 32; mean EDSS score, 1.4) that had not received immunomodulatory drugs or steroids in the 3 mo before blood drawing, a group of IFN-β-treated patients (n = 39; mean EDSS score, 1.78), and a group of patients treated with glatiramer acetate (n = 24; mean EDSS score, 2.0). The study was reviewed and approved by an appropriate institutional review committee.
TIM-3 to regulate T cell function. To do so, we isolated CD4+ T cells from peripheral blood, stimulated them with graded doses of anti-CD3 mAb (1 μg/ml) or an isotype control mouse IgG1 mAb (1 μg/ml; R&D Systems). This TIM-3 Ab has recently been shown to block the functional effects of TIM-3 ligand (galectin-9) binding to TIM-3 (24). Comparable results were obtained using two additional anti-human TIM-3 mAbs that we have generated and characterized (data not shown). PBMCs rather than purified CD4+ T cells were used in some experiments. After 40 h, cells were pulsed with [3H]thymidine and cultured for an additional 18 h, at which point they were harvested on a liquid scintillation counter (PerkinElmer). Before addition of thymidine, supernatants were collected at 40 h for analysis of IFN-γ by ELISA. The sensitivity of the IFN-γ ELISA was 60 pg/ml. IL-17 secretion was measured by ELISA (eBioscience) with a sensitivity of 20 pg/ml.

Cell apoptosis assay

CD4+ T cells from untreated patients with MS and control subjects ex vivo (0 h) or after activation with plate-bound anti-CD3/28 mAbs (both at 1 μg/ml) for 20, 40, and 60 h. In some experiments, CD4+ T cells were isolated from stimulated PBMC cultures by negative isolation using magnetic beads (Miltenyi Biotec). Total RNA was obtained using TRizol reagent (Invitrogen Life Technologies) per the manufacturer’s instructions. Reverse transcription was performed using a First Strand cDNA synthesis kit (Fermentas). The primers and probe for the housekeeping gene GAPDH were obtained commercially (Applied Biosystems). The sequences of primers and probe for human TIM-3 have been described previously (25). Quantitative PCR was performed using an Applied Biosystems PRISM 7500 detection system (PerkinElmer). The amount of target RNA in each sample was normalized to GAPDH according to following equation: \( \text{mRNA} = 2^{-\Delta C_{\text{TIM-3}}} - \Delta C_{\text{GAPDH}} \times 1000 \).

Statistics

All groups were analyzed using Prism software (version 4.0a; GraphPad Software) using the Mann-Whitney U test, a nonparametric test that does not assume Gaussian variation. All of the values were plotted as mean ± SE. \( p \) values <0.05 were considered significant.

Results

TIM-3 blockade during ex vivo CD4+ T cell stimulation enhances IFN-γ secretion in control subjects and treated but not untreated patients with MS

We examined cohorts of healthy controls and subjects with untreated, early relapsing-remitting MS to investigate the ability of TIM-3 to regulate T cell function. To do so, we isolated CD4+ T cells from peripheral blood, stimulated them with graded doses of anti-CD3 mAb in the presence of blocking anti-TIM-3 mAb (24) or isotype control, and then evaluated the functional consequences in terms of proliferation and IFN-γ secretion. As expected, given its role as a negative regulator of IFN-γ-secreting T cells, blocking engagement of TIM-3 increased secretion of IFN-γ in control subjects (Fig. 1A), but did not influence proliferative responses as...
measured by thymidine incorporation. We have observed comparable effects on T cell function using two additional anti-TIM-3 mAbs (data not shown). Similarly, TIM-3 blockade did not alter proliferative responses in untreated patients with MS. In striking contrast to healthy controls, TIM-3 blockade failed to augment IFN-γ secretion during T cell activation in untreated patients. Given reports that IL-17-secreting Th17 cells express TIM-3, although at lower levels than Th1 cells (26, 27), we similarly examined the effects of TIM-3 blockade on IL-17 secretion in a subset of healthy subjects and untreated patients with MS. Consistent with the effects on IFN-γ secretion, TIM-3 blockade enhanced IL-17 secretion in control subjects but not in untreated patients with MS (Fig. 1B).

Similar results with TIM-3 blockade were obtained in independent cohorts of healthy controls subjects and untreated MS patients when PBMCs (rather than purified CD4+ T cells) were stimulated with graded doses of anti-CD3 mAb in the presence or absence of TIM-3 blockade (Fig. 2). Noteworthy is the observation that in the absence of TIM-3 blockade, T cells obtained from untreated MS patients proliferated less extensively than did those obtained from healthy control subjects (p < 0.01 at all doses examined), yet they secreted comparable amounts of IFN-γ. These results suggest that on a per cell basis, T cells present in MS patients secrete greater amounts of IFN-γ than do those from control subjects, which is consistent with past observations (23).

We then examined TIM-3 immunoregulation in patients with MS treated with therapies associated with clinical responses.
These observations confirm results from a recent study that similarly evaluated spontaneous ex vivo apoptosis of T cells present in patients with MS (28).

We next hypothesized that the inability of TIM-3 blockade to enhance IFN-γ secretion was secondary to reduced levels or kinetics of TIM-3 expression after T cell activation. We first attempted to measure TIM-3 expression on ex vivo CD4⁺ T cells using our TIM-3 mAb, but could not detect significant cell surface expression (data not shown). The inability to detect significant numbers of TIM-3⁺ CD4⁺ T cells ex vivo was not due to an inability of the TIM-3 Ab to work in flow cytometric staining since it could readily stain in vitro-polarized Th1 cell lines (data not shown). Therefore, we instead measured TIM-3 mRNA expression using quantitative RT-PCR on ex vivo CD4⁺ T cells and at various time points after T cell stimulation. Consistent with the kinetics of TIM-3 expression in murine T cells, TIM-3 levels increased significantly in stimulated T cells obtained from control subjects at 20 h, with a gradual decline over 60 h (Fig. 5A). In contrast, ex vivo CD4⁺ T cells from untreated MS patients had significantly lower levels of TIM-3 expression at baseline and TIM-3 levels did not increase significantly after 20 h of stimulation; instead, TIM-3 levels gradually increased over the course of 60 h, although they never reached the levels present in stimulated T cells obtained from control subjects. We then performed a similar analysis of the kinetics of TIM-3 expression in T cells obtained from treated patients with MS. Consistent with the ability of IFN-β and glatiramer acetate therapies to restore functional responsiveness to TIM-3 blockade in MS patients, both therapies also restored baseline levels of TIM-3 in ex vivo T cells, and these levels were maintained after T cell activation (Fig. 5B).

To better understand how MS therapies might restore TIM-3 expression on CD4⁺ T cells, purified CD4⁺ T cells or PBMCs were cultured for 48 h in the presence of glatiramer acetate or IFN-β, at which point we isolated RNA and quantitated TIM-3 levels by negative selection from the PBMC cultures before RNA isolation. We observed that neither treatment affected TIM-3 mRNA levels in vitro when added to purified CD4⁺ T cell cultures, but both treatments increased TIM-3 levels on CD4⁺ T cells when added to PBMC cultures (Fig. 6). These data demonstrate that modulation of CD4⁺ TIM-3 expression seen in treated patients can be replicated in vitro and that treatments exert their effects through an APC-dependent process.

**Discussion**

Engagement of mTim-3 down-regulates Th1 responses (20), demonstrating a role in the inhibition of effector Th1 cells during normal immune responses and in the induction of peripheral tolerance (20–22). Thus, we investigated the function of TIM-3 on CD4⁺ T cells isolated directly from the circulation of healthy subjects and patients with MS using newly generated mAbs against human

**FIGURE 4.** TIM-3 blockade does not alter the frequency of apoptotic cells associated with activation-induced cell death. Representative annexin V/propidium iodide staining demonstrates increasing frequencies of annexin V⁺ T cells with increasing doses of anti-CD3 mAb stimulation in both a control subject and untreated MS patient (A). Cumulative data obtained from control subjects (n = 12) and MS patients (n = 9) demonstrates that TIM-3 blockade does not influence the percentages of annexin V⁺ T cells regardless of the strength of T cell activation (B).
TIM-3. Blocking TIM-3 during T cell stimulation significantly enhanced IFN-γ secretion in control subjects but had no effect in untreated patients with MS, demonstrating a defect in TIM-3 immunoregulation that was accompanied by reduced levels and altered kinetics of T cell TIM-3 expression. These data provide a potential mechanistic explanation for the loss of immunoregulation associated with autoreactive T cells in human autoimmune disease.

There are a number of mechanisms by which reduced levels of TIM-3 on circulating CD4+ T cells could contribute to the pathogenesis of MS. Autoreactive T cells with reduced levels of surface TIM-3 expression may be more easily activated and/or resistant to the induction of tolerance, which would serve to both promote and perpetuate a Th1 response. Indeed, our data suggest that enhanced IFN-γ secreted by ex vivo CD4+ T cells obtained from untreated patients with MS may be explained by reduced negative regulation due to reduced levels of TIM-3.

It would be of interest to determine whether there are decreases in TIM-3-expressing CD4+ T cells ex vivo. Because of the low levels of TIM-3 expressed on CD4+ T cells in humans, we examined TIM-3 mRNA levels at the bulk level. Thus, we could not determine whether there are alterations in TIM-3 expression on T cells in untreated and treated patients with MS or whether there are altered frequencies of TIM-3-expressing T cells. However, based on analysis of a large number of CD4+ T cell clones isolated from control subjects and patients with MS, our previous data (23) suggest that TIM-3 levels may be down-modulated on T cells in patients with MS.

IL-17-secreting Th17 cells have been demonstrated in murine models of MS to be highly pathogenic and these cells have been shown to express Tim-3 (26, 27). Thus, altered TIM-3 expression may also influence the regulation of this pathogenic subset of T cells. Our data (Fig. 1B) support this scenario as well. Finally, it has been shown that CD4+ CD25hi regulatory T cells depend on immunoregulation involving TIM-3-dependent and -independent pathways (21). Thus, reduced expression levels of TIM-3 could in part explain the observation that CD4+CD25 regulatory T cell clones from patients with MS are impaired in their ability to suppress T cell proliferation and IFN-γ secretion (29).

The question remains as to why there are altered kinetics of TIM-3 expression on human CD4+ T cells in patients with MS. One possibility is that allelic variants in the TIM-3 pathway or indeed any of the recently discovered MS susceptibility genes (30) may contribute to the lower levels of TIM-3 in patients with MS. Alternately, altered TIM-3 expression and function may be a consequence of disease pathogenesis and activity. Because this study represents ex vivo investigations and a cross-sectional study design, we cannot determine whether alterations in the TIM-3 pathway are primary or secondary in the disease pathogenesis.

Although both glatiramer acetate and IFN-β are effective treatments for some patients with MS, they are effective in only a subset of patients (31). Given that the overwhelming majority of treated patients had levels of TIM-3 expression and function comparable to those of control subjects, it is unlikely that direct modulation of TIM-3 is a mechanism of action of either drug. Nevertheless, these studies open a new pathway for therapeutic intervention where restoration of the TIM-3 signaling pathway in CD4+ T cells from patients with autoimmune disease may correct defects in immunoregulation associated with MS.

Disclosures

The authors have no financial conflict of interest.

References


FIGURE 5. Altered TIM-3 expression in untreated MS patients is restored with therapy. Control subjects (n = 41) rapidly up-regulate TIM-3 expression after stimulation, whereas T cells obtained from untreated patients with MS (n = 28) express lower basal levels of TIM-3 and never up-regulate TIM-3 expression to levels present in control subjects (A). MS patients treated with both glatiramer acetate (n = 19) and IFN-β (n = 29) express basal levels of TIM-3 comparable to those of control subjects (B). Expression levels after stimulation remain comparable to those of control subjects (data not shown). Treated MS patients and control subjects express significantly higher levels of TIM-3 (p < 0.01) at all time points examined.

FIGURE 6. In vitro treatment with glatiramer acetate and IFN-β can up-regulate TIM-3 on CD4+ T cells. Purified CD4+ T cells or PBMCs were cultured in the absence or presence of glatiramer acetate (10 μg/ml) or IFN-β (1000U/ml). Total RNA was isolated and mRNA levels of TIM-3 were determined by quantitative RT-PCR. Importantly, CD4+ T cells were isolated by negative selection from PBMC cultures before RNA isolation. Error bars represent SE based on samples from six healthy subjects.


