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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.

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.
The blockade had no effect on IFN-γ production from stimulated ex vivo CD4+ T cells from control subjects \((n = 37)\), with no effect on proliferative responses. In contrast, TIM-3 blockade had no effect on IFN-γ secretion from CD4+ T cells isolated from untreated MS patients \((n = 18; A)\). The effects of blocking anti-TIM-3 mAb on IL-17 secretion were similarly examined in a subset of control subjects \((n = 5)\) and untreated patients with MS \((n = 5; B)\).

FIGURE 1. Lack of TIM-3 immunoregulation in untreated patients with MS. The presence of blocking anti-TIM-3 mAb augments IFN-γ production from stimulated ex vivo CD4+ T cells from control subjects \((n = 37)\), with no effect on proliferative responses. In contrast, TIM-3 blockade had no effect on IFN-γ secretion from CD4+ T cells isolated from untreated MS patients \((n = 18; A)\). The effects of blocking anti-TIM-3 mAb on IL-17 secretion were similarly examined in a subset of control subjects \((n = 5)\) and untreated patients with MS \((n = 5; B)\).

**T cell stimulation**

PBMCs from MS patients or control subjects were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Technology), and CD4+ T cells were subsequently isolated by negative selection using magnetic beads (>90% purity, CD4 isolation kit II; Miltenyi Biotec). T cells \((5 \times 10^5/well)\) were then cultured in 96-well plates precoated with graded doses of anti-human CD3 \((0–1.25 \mu g/ml, clone HIT3a)\) and anti-human CD28 \((0.5 \mu g/ml, clone CD28.2) mAbs (BD Biosciences) in the presence of an anti-human TIM-3 mAb \((1 \mu g/ml)\) or an isotype control mouse IgG1 mAb \((1 \mu g/ml; R&D Systems)\). 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 \(^{3}H\)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 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 (Fermentals). 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: RNA = \(2^{-\Delta\Delta C_T(TIM-3)} = \Delta C_T(GAPDH) \times 1000\).

**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 \(\pm\) 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
The presence of blocking anti-TIM-3 mAb augments IFN-γ production from stimulated ex vivo PBMCs from control subjects (n = 12), with no effect observed when examining PBMCs obtained from untreated MS patients (n = 3). In contrast, the presence of anti-TIM-3 mAb enhanced IFN-γ secretion in treated MS patients (glatiramer acetate, n = 2; IFN-β, n = 5). We then examined TIM-3 immunoregulation in patients with MS treated with therapies associated with clinical responses. Whereas TIM-3 blockade was unable to enhance IFN-γ secretion from activated CD4⁺ T cells in untreated patients, the effects of TIM-3 blockade were apparent in patients treated with IFN-β and glatiramer acetate (Fig. 3A). We examined the influence of the strength of signal delivered through the TCR on the ability of TIM-3 blockade to enhance IFN-γ secretion and observed that TIM-3 blockade had its greatest effect when T cells received weaker strengths of signal (lower doses of anti-CD3 mAb stimulation) (Fig. 3B). This observation was true for untreated and treated patients that received both types of therapy, as well as for control subjects. The less pronounced effect of TIM-3 blockade at higher strengths of TCR stimulation is likely due to difficulty in completely blocking the higher levels of TIM-3 associated with stronger TCR stimulation.

Reduced TIM-3 expression can explain lack of TIM-3 immunoregulation in untreated patients with MS and is corrected with treatment

To examine the mechanism responsible for the lack of TIM-3 immunoregulation in untreated MS patients, we examined the extent of apoptosis associated with T cell stimulation from untreated MS patients and control subjects in the presence of blocking anti-TIM-3 mAb or isotype control. We speculated that TIM-3 blockade might protect T cells from galectin-9/TIM-3-mediated apoptosis, leading to the enhanced IFN-γ secretion seen in control subjects. We failed to observe any significant influence of TIM-3 blockade on the percentage of apoptotic cells in untreated MS patients or control subjects at all concentrations of anti-CD3 mAb stimulation (Fig. 4A). Interestingly, we did observe that T cells obtained from untreated patients with MS were more prone to apoptosis than those from control subjects (p < 0.05; Fig. 4B).
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 quantitative RT-PCR; CD4+ T cells were purified 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
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+ CD25^{high} regulatory T cell-dependent immunoregulation involves TIM-3-dependent and -independent pathways (21). Thus, reduced expression levels of TIM-3 could in part explain the observation that CD4+ CD25^{high} 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