A role for the TIM-3/GAL-9/BAT3 pathway in determining the clinical phenotype of multiple sclerosis

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ABSTRACT T-cell immunoglobulin and mucin domain 3 (Tim-3) ligation of galectin-9 (Gal-9); this process, resulting in the inhibition of T\textsubscript{h}1 responses and in the apoptosis of antigen-specific cells, is hampered by binding of the molecular adaptor human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) to the intracellular tail of Tim-3. Apoptosis of myelin basic protein (MBP)-specific T lymphocytes correlates with reduced rates of disease progression in multiple sclerosis (MS). We extensively analyzed the Tim-3/Gal-9/Bat3 pathway in 87 patients with a diagnosis of stable relapsing-remitting MS (RRMS), primary progressive MS (PPMS), or benign MS (BEMS), as well as in 40 healthy control (HC) subjects. Results showed that MBP-specific CD4\textsuperscript{+}Tim-3\textsuperscript{+}, CD4\textsuperscript{+}/Gal-9\textsuperscript{+}, and CD4\textsuperscript{+}/Tim-3\textsuperscript{+}/AV\textsuperscript{+} (apoptotic) T lymphocytes were augmented in the BEMS group, whereas CD4\textsuperscript{+}/Bat3\textsuperscript{+} and CD8\textsuperscript{+}/Bat3\textsuperscript{+} T lymphocytes were increased and CD4\textsuperscript{+}/Tim-3\textsuperscript{+}/AV\textsuperscript{+} T cells were reduced in the PPMS group (>2 fold and \textit{P}<0.05 in all cases). Blocking the Tim-3/Gal-9 interaction with specific mAb reduced T-lymphocyte apoptosis and augmented production of IFN\textgamma and IL-17 in the BEMS, RRMS, and HC groups, but not in the PPMS group. The Tim-3/Gal-9 interaction favors apoptosis of MBP-specific T lymphocytes in BEMS; this process is reduced in PPMS by the up-regulation of Bat3. Therapeutic interventions aimed at silencing Bat3 could be beneficial in MS.—Saresella, M., Piancone, F., Marventano, I., La Rosa, F., Tortorella, P., Caputo, D., Rovaris, M., Clerici, M. A role for the Tim-3/Gal-9/Bat3 pathway in determining the clinical phenotype of multiple sclerosis. \textit{FASEB J.} 28, 5000–5009 (2014). www.fasebj.org

Key Words: apoptosis • myelin basic protein • T cell

T-CELL IMMUNOGLOBULIN AND MUCIN DOMAIN 3 (Tim-3) is expressed in humans on a subset of activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes, on differentiated T-helper 1 (T\textsubscript{h}1) cells, and at lower levels on T\textsubscript{h}17 lymphocytes (1), as well as in cells of the innate immune system, including mast cells (2), macrophages and dendritic cells (DCs) (3), natural killer (NK) and natural killer T (NKT) cells (4), and monocytes (3). The expression of this protein is regulated by the T\textsubscript{h}1-associated transcription factor T-bet, and reduced Tim-3 levels are seen in T-bet-deficient mice (5). Tim-3 binds galectin-9 (Gal-9), a member of the S-type lectins whose expression is also regulated by T-bet (6–9), which is present in lymphocytes and, according to the local cytokine milieu, in endothelial cells and fibroblasts.

Tim-3 on DCs synergizes with Toll-like receptors to promote TNF-\alpha secretion, promoting T\textsubscript{h}1-cell responses. Tim-3 is then expressed by T\textsubscript{h}1 cells that have differentiated into T\textsubscript{h}1, and this favors the T-bet-mediated up-regulation of Gal-9 (2, 5) and the production of IFN\gamma (1, 10). At this point, the engagements of Tim-3 by Gal-9 result in intracellular calcium flux and T\textsubscript{h}1-lymphocyte death within a regulatory feedback mechanism (5, 1115); notably, T\textsubscript{h}17 lymphocytes are less susceptible to Gal-9-mediated cell death as a consequence of their lower Tim-3 expression levels (1, 10, 16, 17). Recent results have shown that Gal-9-mediated apoptosis can be prevented by molecular adaptor human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3), a cellular factor that binds the intracellular tail of Tim-3 and promotes both proliferation and proinflammatory cytokine production (18, 19).

Abbreviations: AV, annexin V; Bat3, molecular adaptor human leukocyte antigen B (HLA-B)-associated transcript 3; BE, benign multiple sclerosis; CNS, central nervous system; EAE, experimental autoimmune encephalitis; EDSS, Expanded Disability Status Scale; Gal-9, galectin-9; HC, healthy control; mAb, monoclonal antibody; MBP, myelin basic protein; MFI, mean fluorescence intensity; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PPMs, primary progressive multiple sclerosis; RA, rheumatoid arthritis; RRMS, relapsing-remitting multiple sclerosis; T\textsubscript{h}, T helper; Tim-3, T-cell immunoglobulin and mucin domain 3

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Tim-3 alterations hamper the apoptosis of antigen-specific T<sub>H</sub>1 cells and play a role in chronic autoimmune diseases, including multiple sclerosis (MS) and rheumatoid arthritis (RA) (20, 21). In MS, in particular, it was shown that Tim-3 expression correlates with IFNγ and TNF-α concentration in the cerebrospinal fluid (CSF) of patients with an active form of disease (4). CSF-derived T-cell clones express lower levels of Tim-3 compared with those of healthy controls (HCs; ref. 20); reduced levels of Tim-3 expression in T lymphocytes can be restored by therapy (22); and monocytes and microglia from the active border regions of MS lesions express higher levels of Tim-3 than those from the quiescent center of lesions (3). Additional results showed that Gal-9 is significantly elevated on the astrocytes present in MS lesions compared to the levels seen in normal human central nervous system (CNS) tissue (5).

In experimental autoimmune encephalitis (EAE), the archetypal model of MS, administration of Tim-3 monoclonal antibody (mAb) during the induction phase worsens disease progression and leads to the accumulation of activated macrophages (23) and a significant demyelination of spinal cord neurons. Gal-9 mAb in the same phase of disease diminishes the number of IFNγ-producing cells, reducing disease severity (13), whereas in vivo knockout of Gal-9 results in the exacerbation of myelin oligodendrocyte glycoprotein (MOG)35–55-driven EAE (13). In the same model, recent data demonstrated that mice lacking Bat3 develop a less severe form of EAE than wild-type controls (24). Even more recently, the prevention of Tim-3 signaling in CD4<sup>+</sup> T cells-induced EAE was shown to alter the CNS inflammatory pattern due to a differential effect on T<sub>H</sub>1 vs. T<sub>H</sub>17 cells, whereas preventing Tim-3 signaling in CD8<sup>+</sup> T-cell-mediated EAE exacerbated disease (24).

Apoptosis of myelin basic protein (MBP)-specific T lymphocytes was suggested to be a protective mechanism against the progression of MS (25–26). Because the Tim-3/Gal-9 interaction stimulates the apoptosis of antigen-specific T<sub>H</sub>1 cells and Bat3 protects such cells from Gal-9-mediated cell death, we thought it interesting to analyze the possible role of these molecules and pathways in patients with different forms of MS.

MATERIALS AND METHODS

Patients and control participants

Eighty-seven patients affected by MS as diagnosed by clinical and laboratory parameters and followed by the Centro Sclerosi Multipla of the Don Gnocchi Foundation (Milan, Italy) were included in the study. Stable disease was diagnosed in 30 patients with relapsing-remitting MS (RRMS; median age 40 yr; range 20–59 yr; 19 females and 11 males) on the basis of brain and spinal cord magnetic resonance imaging (MRI), with gadolinium showing no areas of enhancement at the time of enrollment. Median disease duration was 7 yr (range 1–29 yr); the median Kurtze Expanded Disability Status Scale (EDSS) score was 1.5 (range 1–6). Twenty-four patients (median age 49 yr; range 32–67 yr; 12 females and 12 males) were diagnosed as being affected by primary progressive MS (PPMS) on the basis of the clinical history and of brain and spinal cord MRI with gadolinium that evidenced a stability of the lesion load at the time of enrollment. Median disease duration was 20 yr (range 6–35 yr); the median EDSS was 6.5 (range 4.5–8). Finally, 33 patients (median age 45 yr; range 36–61 yr; 22 females and 11 males) were affected by benign MS (BEMS) as confirmed by brain and spinal cord MRI with gadolinium; MRI showed stability or, in many cases, an improvement of the lesion load at the time of enrollment. BEMS was determined to be present based on the most widely accepted definition, i.e., an EDSS score ≤ 3.0 (meaning that the patient is fully ambulatory but may have moderate disability in one functional system or mild disability in up to 4 functional systems) after ≥15 yr from the clinical onset of disease. Median disease duration was 21 yr (range 15–30 yr); median EDSS score was 2 (range 0–3). None of the patients had received immunosuppressive drugs in the year prior to the study period; all of them gave informed consent, according to a protocol approved by the local ethics committee of the Don Gnocchi Foundation. Finally, a group of 40 sex- and age-matched HC participants (median age 48 yr; range 33–62; 27 females and 13 males) was enrolled in the study.

Blood sample collection and cell separation

Whole blood (30 ml) was collected in Vacutainer tubes containing EDTA (Becton Dickinson & Co., Rutherford, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were separated on lymphocyte separation medium (Organon Teknika Corp., Durham, NC, USA) and washed twice in PBS. Leukocytes viability was determined using a Scepter Hand- Automated Cell Counter (Millipore, Boston, MA, USA).

Stimulation of PBMCs for flow cytometry analysis

PBMCs resuspended at 1 × 10<sup>6</sup> cells/ml were stimulated with nonimmunogenic peptides (27) or with a pool of antigenic MBP peptides (10 μg/ml; ref. 27) + anti-CD28 mAb (1 μg/ml; clone 37407.111; R&D Systems, Minneapolis, MN, USA) to facilitate costimulation, at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 24 h. For cytokine analyses, brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the cell cultures during the last 6 h of stimulation to block protein secretion.

Immunofluorescent staining

PBMCs were stained with CD4<sup>+, </sup>CD8<sup>+, </sup>CD14<sup>+, </sup>and Tim-3-specific mAbs for 30 min at room temperature in the dark. For the analysis of cytokine-secreting or intracellular protein-expressing cells, PBMCs were washed and fixed (Fix and Perm cell permeabilization kits; Caltag Laboratories, Burlingame, CA, USA) for 15 min at room temperature in the dark. Cells were then washed and resuspended in permeabilization reagent (Fix and Perm kits)) with Bat3-, Gal-9-, TNF-α, IFNγ-, or IL17A-specific mAbs. After incubation, cells were washed and fixed in 1% paraformaldehyde in PBS.

Evaluation of apoptosis

PBMCs were stained with CD4<sup>+, </sup>CD8<sup>+, </sup>and Tim-3-specific mAbs for 30 min at 4°C in the dark. For the apoptosis assay, PBMCs were then washed and resuspended in ice-cold 1× binding buffer (Beckman-Coulter, Fullerton, CA, USA) plus...
10 μl of annexin V (AV) and 20 μl of 7-AAD viability dye for 15 min on ice in the dark. Finally, cell suspensions were resuspended in 400 μl of 1× binding buffer and analyzed by flow cytometry. Notably, the use of 7-AAD, a DNA-specific viability dye, allows early apoptotic cells to be distinguished from late-apoptotic and necrotic cells. To this goal, a gate capable of distinguishing early apoptosis events (AV<sup>+</sup>high and 7 AAD<sup>+</sup>) was selected.

**Blocking assay**

Purified anti-human Gal-9 (mouse IgG1k, clone 9M1-3; Biolegend, San Diego, CA, USA) or anti-Tim-3 (mouse IgG1k, clone F38-2E2; Biolegend) mAbs were added (10 μg/ml) to cell cultures together with the MBP peptide pool and left throughout the stimulation period. Mouse IgG1 isotype control antibodies were used in MBP-stimulated cell cultures.

**mAbs**

The following mAbs were used in this study: phycoerythrin (PE)-labeled anti-human Tim-3 (rat IgG2A; clone 344823; R&D Systems), allophycocyanin (APC)-labeled anti-human Tim-3 (rat IgG2A; clone 344823; R&D Systems), PE-labeled anti-human Gal-9 (mouse IgG1; clone 9M1-3; Biologend, PE-cytox-7 (PC7)-labeled anti-CD4 (clone SFC12T4D10; mouse IgG2A; Beckman-Coulter), PE-cytox-5 (PC5)-labeled anti-CD8 (clone B9.11; mouse IgG1; Beckman-Coulter), FITC or PC5-labeled anti-CD14 (clone 116; mouse IgG1; Beckman-Coulter), anti-human AV-FITC (mouse-IgG1; Beckman-Coulter). Intracellular proteins were stained with the following mAbs: anti IFNγ-FITC (clone 4S.B3; mouse IgG1k; eBioscience, San Diego, CA, USA), anti-TNF-α-carboxyfluorescein (CFS; clone 6401; mouse IgG1; R&D Systems), anti-IL-17A-FITC (clone eBio64DEC17; mouse IgG1k; eBioscience). Finally, anti-Bat3 (clone 2B21; mouse IgMk; Abnova Taipei, Taiwan) was conjugated using the Lightning-Link R-PE conjugation kit (Innova Biosciences, Cambridge, UK).

**Cytometric analysis**

Analyses were performed using a Beckman-Coulter Gallios flow cytometer equipped with two lasers operating at 488 and 638 nm, respectively, interfaced with Gallios software and analyzed with Kaluza v 1.2; 200,000 events were acquired and gated on forward- and side-scatter properties for lymphocytes or monocytes. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1–FL6. Samples were first run using isotype control or single fluorochrome-stained preparations for color compensation. Rainbow calibration particles (Spherotec, Lake Forest, IL, USA) were used to standardize results.

**Statistical analysis**

Quantitative data were not normally distributed (Shapiro-Wilk test) and are thus summarized as median and interquartile range (25th and 75th percentiles). Comparisons between groups were evaluated to identify immunological differences. Kruskal-Wallis ANOVA was performed for each variable. Comparisons among the different groups were made using a 2-tailed Mann-Whitney U test performed for independent samples. Data analysis was performed using the MedCalc statistical package (MedCalc Software bvba, Mariakerke, Belgium).

**RESULTS**

**MBP-stimulated CD4<sup>+</sup>, CD8<sup>+</sup>, and CD14<sup>+</sup> cells expressing Tim-3**

Tim-3-expressing immune cells were analyzed in MBP-stimulated and in control peptide-stimulated PBMCs of all patients and HC participants by flow cytometry. Whereas no differences were detected when cells were incubated with the pool of control, nonantigenic peptides (Fig. 1), MBP-stimulated Tim-3-expressing CD4<sup>+</sup> T lymphocytes (CD4<sup>+</sup>/Tim-3<sup>+</sup> cells) were significantly augmented in patients with BEMS compared to all the other groups of patients (P=0.001 vs. PPMS; P=0.05 vs. RRMS) and HC participants (P=0.007), with the lowest percentages of these cells being observed in individuals with CD8<sup>+</sup>/Tim-3<sup>+</sup> cells were also significantly increased in patients with BEMS and RRMS compared with both patients with PPMS (P=0.0003 and P=0.01, respectively) and HC participants (P=0.0003 for both). Finally, Tim-3-expressing CD14<sup>+</sup> cells were comparable in all the groups analyzed (Fig. 1).

Results obtained on analyzing mean fluorescence intensity (MFI) showed that Tim-3 MFI was significantly augmented, as well, in CD4<sup>+</sup> and CD8<sup>+</sup> T cells of patients with BEMS, with the lowest values seen in lymphocytes of patients with PPMS (Fig. 2).

**Gal-9 in MBP-stimulated CD4<sup>+</sup>, CD8<sup>+</sup>, and CD14<sup>+</sup>**

Similar percentages of Gal-9<sup>+</sup> T lymphocytes were seen in all the groups analyzed on incubation with control peptides (Fig. 3); nevertheless, significant differences emerged in MBP-stimulated cells. Thus, CD4<sup>+</sup>/Gal-9<sup>+</sup> T lymphocytes were augmented in the BEMS, RRMS, and HC groups compared to the PPMS group (P=0.008, 0.03, and 0.01, respectively). Also, in this case, the lowest quantities of CD4<sup>+</sup>/Gal-9<sup>+</sup> T lymphocytes in MBP-stimulated cell cultures were seen in cells of patients with a diagnosis of PPMS. Similar trends, that nevertheless did not reach statistical significance, were observed in MBP-stimulated CD8<sup>+</sup> and CD14<sup>+</sup> cells (Fig. 3).

**MBP-stimulated apoptotic CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes**

The interaction between Tim-3 and Gal-9 results in the apoptosis of antigen-specific T<sub>h</sub> cells. Apoptosis of cells incubated with either MBP or the control peptides was evaluated next by analyzing CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes that coexpress Tim-3 and AV in patients with MS and HC participants. Results indicated that, whereas once again no differences were seen when cells were incubated with the control peptides, MBP-stimulated CD4<sup>+</sup>/Tim-3<sup>+</sup>/AV<sup>+</sup> (apoptotic) T lymphocytes were significantly increased in the BEMS, RRMS, and HC groups compared to patients with PPMS (P=0.02, 0.02, and 0.005, respectively), in whom the lowest amounts of apoptotic CD4<sup>+</sup> T lymphocytes were detected (Fig. 4C). MBP-stimulated CD8<sup>+</sup>/Tim-3<sup>+</sup>/AV<sup>+</sup> lymphocytes were marginally increased in patients with BEMS, as well, but the differences were not significant (Fig. 4D).
Analyses performed adding a fourth marker (IFNγ or IL-17) showed that even if higher quantities of MBP-stimulated CD4+/Tim-3+ and CD8+/Tim-3+ T lymphocytes were seen in patients with BEMS, the differences were not significant (data not shown).

Incubation of cell cultures prior to MBP stimulation with either Tim-3- or Gal-9-specific blocking mAbs reduced the percentage of CD4+/AV− and CD8+/AV− T lymphocytes in all the groups analyzed, with the exception of patients with PPMS, in whom apoptotic cells were instead increased on incubation with both antibodies (Fig. 4E, F). These results confirm that the interaction between Tim-3 and Gal-9 induces apoptosis of antigen-specific lymphocytes and indicate that this pathway is not defective per se in MS, with the exception of what occurs in patients affected by the particularly severe progressive form of disease (PPMS).

Figure 1. MBP-stimulated Tim-3-expressing CD4+, CD8+, and CD14+ cells. A–D) MBP-stimulated PBMCs stained with Tim-3 isotype control (A, C) or with TIM-3- and CD4-specific (B) or CD8-specific (D) mAbs. Representative results in patients with PPMS (PP), BEMS (BE), or RRMS (RR) and in a HC participant are presented. Top right corners show the percentage of CD4+/Tim-3+ and CD8+/Tim-3+ T cells relative to the total number of lymphocytes. E–G) Summary results on stimulation of CD4+/Tim-3+ (E), CD8+/Tim-3+ (F), or CD14+/Tim-3+ (G) cells with nonimmunogenic peptides (solid line) or MBP (dashed line). Boxes stretch from the 25th to the 75th percentile; lines across boxes indicate median values; lines stretching from boxes indicate extreme values. Statistical significance is shown.

Figure 2. Mean fluorescence intensity (MFI) of Tim-3 on MBP-stimulated CD4+ and CD8+ T cells. Summary results in CD4+ T cells (A) and CD8+ T cells (B) from patients with PPMS (PP), BEMS (BE), or RRMS (RR) and HC participants. Boxes stretch from the 25th to the 75th percentile; lines across boxes indicate median values; lines stretching from boxes indicate extreme values. Statistical significance is shown.
Bat3-expressing CD4⁺ and CD8⁺ T cells in MBP-stimulated PBMCs

Binding of the molecular adaptor Bat3 to the intracellular tail of Tim-3 protects lymphocytes from Tim-3/Gal-9-mediated cell death and stimulates the preferential production of IFNγ by Treg cells. Bat3 intracellular expression was analyzed next in control peptides and in MBP-stimulated cells of all the individuals enrolled in the study. The lowest percentage of Bat3-expressing CD4⁺ T lymphocytes was seen in patients with BEMS, whereas the highest amount of CD4⁺/Bat3⁺ cells was observed in patients with PPMS. The increase seen in patients with PPMS was significant compared with the results obtained in all the other groups (P=0.003 vs. BEMS; P=0.001 vs. RRMS, P=0.02 vs. HC). Notably, similar results were observed when CD8⁺ Bat3⁺ cells were analyzed, as these cells were statistically augmented in patients with PPMS compared to the RRMS and HC groups (P=0.02 in both cases; Fig. 5E, F). Also, in this case, no differences were detected when PBMCs were incubated with control peptides (Fig. 5).

IFNγ- and IL-17-expressing T lymphocytes: effect of Tim-3- and Gal-9-blocking antibodies

IFNγ- and IL-17-producing CD4⁺ and CD8⁺ T lymphocytes were analyzed in MBP-stimulated cultures after incubation with Tim-3- or Gal-9-blocking mAbs. In both cases, incubation resulted in increased percentages of MBP-stimulated CD4⁺/IFNγ⁺ and CD8⁺/IFNγ⁺ T lymphocytes in the BEMS, RRMS, and HC groups, whereas these cells were reduced in the PPMS groups (Fig. 6A). Similar results were obtained when IL-17-producing cells were analyzed. Thus, incubation with Tim-3- or Gal-9-blocking antibodies augmented MBP-stimulated CD4⁺/IL-17⁺ and CD8⁺/IL-17⁺ lymphocytes in all groups of individuals, with the exception of patients with PPMS, in whom these cells were instead reduced (Fig. 6B).

Because Tim-3- and Gal-9-blocking antibodies impede the reciprocal ligation of these receptors, thus reducing apoptosis, and, as indicated above, in vitro apoptosis is down-modulated by incubation of cells with these antibodies in the BEMS, RRMS, and HC groups but not in patients with PPMS, the increased percentage of IFNγ and IL-17-expressing T lymphocytes seen in the BEMS, RRMS, and HC groups is likely to be the consequence of a reduced apoptosis rate.

DISCUSSION

Binding of Tim-3 to Gal-9 induces the apoptosis of antigen-specific cells; this process is reduced by the ligation of Bat3 to the intracellular tail of Tim-3 (5, 11–15, 18, 19). Because MBP-specific T lymphocytes are
suggested to play a direct pathogenic role in MS, and their apoptosis was observed to be associated with reduced or absent rates of disease progression (25–26), we analyzed the possible involvement of the Tim-3/Gal-9/Bat3 pathway in patients with different MS phenotypes. Results indicated that increased Tim-3 and Gal-9 expression is seen in individuals with a diagnosis of BEMS, resulting in an augmented death rate of MBP-specific CD4<sup>+</sup>/H<sub>11001</sub>T lymphocytes. This was confirmed by the observation that Bat3-expressing cells are reduced in these same individuals. Because Bat3 impedes the Tim-3/Gal-9-mediated apoptosis (18, 19), its reduction in BEMS offers an additional justification for the increased susceptibility to apoptosis of MBP-specific cells seen in these patients. Notably, the situation observed in PPMS, a particularly aggressive form that results in rapid disease progression, was drastically different. Thus, in these individuals, a reduced quantity of Tim-3<sup>+</sup> and Gal-9<sup>+</sup> T lymphocytes, as well as an increased amount of Bat3-expressing cells, was associated with a significantly reduced rate of apoptosis of MBP-specific cells.

It could be argued that the frequencies of cells expressing Tim-3, Gal-9, and Bat3 are extremely low; it is, nevertheless, important to underline that what are shown herein are the frequencies of MBP-stimulated cells. We are, thus, specifically considering and analyzing only those antigen-specific cells that are suspected to drive the disease. In the light of this consideration of the realization that the percentage of lymphocytes for any specific antigen in peripheral blood is below detection, and of the literature showing how, in other diseases, the analysis of apparently minuscule quantities of antigen-specific cells correlates with clinical endpoints (28), this critique is not justified.

Circulating autoreactive T cells that attack and damage self-antigens in autoimmune diseases originate from alterations in the complex mechanisms of clonal deletion taking place during central tolerance (29). These cells also manage to elude peripheral tolerance mechanisms, including induction of anergy and/or apoptosis (30). If both control mechanisms are avoided, self-reactive T cells are activated, and autoimmune responses are initiated (31). In MS, autoreactive T lymphocytes specific for MBP play an important pathogenic role; these cells are activated and clonally expanded in the disease, and they accumulate in the CNS, as well as in peripheral compartments (32–33).
Apoptosis of MBP-specific cells was observed to be beneficial in MS by reducing the size of the pool of auto-reactive T lymphocytes (25–26). Apoptosis can be mediated by a number of mechanisms, among which the interaction between proteins expressed on cell surface has a prominent importance. We have previously focused on the programmed cell death (PD)1/PD-L1-mediated cell death and have shown that this pathway is greatly deficient in RRMS during the phases of disease activity (26). Data herein offer further support to the concept that alterations in the mechanisms that control the apoptotic rate of antigen-specific cells are involved in the pathogenesis of autoimmune conditions. Thus, results indicating that the Tim-3/Gal-9-driven apoptosis of MBP-specific T cells is significantly augmented in patients with a diagnosis of BEMS, a disease phenotype in which lack of apparent disease progression associates with a marginal degree of inflammation, and a MRI-documented stability or improvement of the lesion load suggest a role for this immune pathway in determining disease progression. This hypothesis is further strengthened by the specular results obtained on analyzing this pathway in PPMS, an aggressive form of MS that is at the opposite end of the clinical spectrum of disease manifestation.

Our results confirm previous observations showing the presence of a dysregulation of Tim-3 expression in lymphocytes of patients with MS (20, 22). Data herein are also the first report of an alteration of Bat3 levels in individuals with MS and lend support to recent results indicating that increased Bat3 expression is associated with the promotion of autoimmunity, possibly as a consequence of its ability to obstacle Tim-3-mediated apoptosis (18). Finally, results showing that increased amounts of Tim-3/H11001 cells that coexpress AV (apoptotic lymphocytes) were increased in BEMS are in accordance with data obtained both in the EAE model and in patients, indicating that Tim-3-mediated apoptotic signaling is involved in preventing the spread of autoreactive T cells (3–4, 13, 18, 20, 23).

Experiments in which the Tim-3/Gal-9 interaction was impeded by blocking antibodies resulted in a reduction of apoptotic MBP-specific T lymphocytes in the BEMS, RRMS, and HC groups; these cells were increased instead in patients with PPMS. The use of Tim-3- and Gal-9-blocking antibodies also resulted in an
increased percentage of MBP-specific IFNγ- and IL-17-producing cells in all individuals, again with the exception of patients with PPMS. These results confirm that the Tim-3/Gal-9 interaction mediates apoptosis of IFNγ- and IL-17-producing T lymphocytes and indicate that this pathway is altered in PPMS, suggesting a T-cell-intrinsic deregulation of both Tim-3 expression and function in this subset of patients with MS. Notably, results of experiments performed in the mouse model showed that whereas higher concentrations of Gal-9 result in the apoptosis of Tim-3-expressing Th1 and Th17 cells, reduced amounts of this protein favor the proliferation of Th1 lymphocytes (34). This seems to be the situation observed in patients with PPMS, in whom very low amounts of Gal-9 are associated with low apoptotic rates and higher percentages of MBP-specific Th1 and Th17 cells.

Comparisons between levels of Tim-3 expression in Th1 and Th17 lymphocytes have shown lower levels of Tim-3 expression in Th17 cells of both mice (10, 16) and humans (1). These data led to the suggestion that low-level Tim-3 expression may allow escape of Th17 cells from Gal-9-mediated cell death (17). The biological consequences of the low Tim-3 expression by Th17 cells have, nevertheless, not been established. Our results suggest that the levels of Tim-3 and Gal-9 expressed by cells of patients with RRMS and BEMS are sufficient to warrant the functionality of the Tim-3/Gal-9 pathway. This pathway is malfunctioning in patients with PPMS, probably because of the reduced levels of both proteins observed in cells of these individuals or, alternatively, as a consequence of alterations in their functionality. That functional alterations could be responsible for the reduced rate of apoptosis of MBP-specific cells in patients with PPMS is reinforced by the observation that the expression levels of these proteins are similar in patients with PPMS and HC participants; nevertheless, in HC participants, the percentage of antigen-specific apoptotic lymphocytes is significantly increased.

Dysregulation of Tim-3 expression is not limited to MS, as it has been previously reported both in psoriasis (35) and in RA (36–37). In RA, in particular, higher Tim-3 expression was shown to correlate with lower indexes of disease activity, a picture that is very similar to what we observed in stable RRMS and BEMS. Notably, Tim-3 expression on T lymphocytes is up-regulated in patients with MS who are treated with either IFN or glatiramer acetate (22); similarly, increased Tim-3 expression is described in patients with RA who enter remission after treatment (36).

Recent evidence in chronic viral infections indicates that expression of Tim-3 marks a population of exhausted T cells (38–40). Exhaustion of antigen-

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**Figure 6.** Blockade of the Tim-3/Gal-9 pathway. MBP-stimulated CD4+ and CD8+ T cells producing INF-γ (A) or IL17A (B). Data obtained after MBP stimulation and on blocking the Tim-3/Gal-9 interaction with Tim-3- or Gal-9-specific antibodies are shown. Mean values are presented: *P < 0.05 vs. MBP.
specific lymphocytes is a negative occurrence in viral infections, but could be beneficial in the case of autoimmune conditions, diseases in which exhaustion would lead to disease improvement because of the reduction of the pool of autoreactive cells (41).

In this light, the observations that successful therapy results in an increased expression of Tim-3 (42–44) could be the consequence of a higher number of exhausted lymphocytes. It will be interesting to verify this hypothesis in follow-up studies.

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