Costimulatory Pathways in Multiple Sclerosis: Distinctive Expression of PD-1 and PD-L1 in Patients with Different Patterns of Disease

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Costimulatory Pathways in Multiple Sclerosis: Distinctive Expression of PD-1 and PD-L1 in Patients with Different Patterns of Disease

Daria Trabattoni,²* Marina Saresella,²† Michela Pacei,* Ivan Marventano,† Laura Mendoza,‡ Marco Rovaris,‡ Domenico Caputo,‡ Manuela Borelli,* and Mario Clerici ³†§

T lymphocytes costimulatory molecules, including CD80, CD86, CD28, CTLA4, PD-1, PD-L1, and B7-H3, are associated with the preferential expression of pro- or anti-inflammatory cytokines. We analyzed the expression of these molecules and myelin basic protein (MBP)-specific IL-10 and IFN-γ production in patients with multiple sclerosis (MS) with relapsing-remitting acute (AMS, n = 40) or stable (SMS, n = 38). Twenty-two patients successfully undergoing therapy with glatimer acetate (n = 12) or IFNβ (n = 10) were also analyzed. MBP-specific and PD-1-expressing T lymphocytes, PD-L1-expressing CD19⁺ cells, and PD-L1⁺/IL-10⁺/CD14⁺ and CD19⁺ cells were significantly augmented in SMS patients. Additionally, MBP-specific and annexin V-expressing CD4⁺ and CD8⁺ (apoptotic) T lymphocytes were augmented and pAkt-positive (proliferating) cells were decreased in SMS compared with AMS patients. PD-1 ligation resulted in the increase of pAkt⁺ lymphocytes in AMS patients alone. B7-H3 expression and IFN-γ production were comparable in all individuals but the PD-L1⁺/IL-10⁺ over B7-H3⁺/IFN-γ⁺ ratio was significantly lower in AMS compared with SMS patients. Finally, PD-L1 expression on immune cells was reduced in treated patients, suggesting that therapy-induced disease remission is not associated with the modulation of the expression of this molecule. The PD-1/PD-L1 pathway plays an important role in modulating immune functions in MS patients; monitoring and targeting these proteins could offer diagnostic and therapeutic advantages.


Abstract

Cell activation is a complex and multistep phenomenon resulting from the recognition by the TCR of a peptide/MHC molecule complex on the surface of APCs (1). Optimal activation is nevertheless only achieved upon the interaction between pairs of costimulatory molecules that are situated on the surfaces of both APC and T lymphocytes. These interactions are not Ag-specific, as costimulatory molecules are by definition non-polymorphic and invariant, but the interaction between different molecules modulates both the amplitude and the quality of the ensuing immune response (2, 3). A pivotal role in T lymphocytes stimulation is played by the B7 family of costimulatory molecules. This family includes a growing number of different proteins that are characterized by a single-chain glycoprotein that presents two extracellular Ig-like domains as well as a transmembrane segment and an intracytoplasmic domain (4). Among these molecules the share the ability to regulate T cell activation (4, 5). The best described B7 molecules, B7-1 (CD80) and B7-2 (CD86), are expressed on APC and engage proteins belonging to the CD28 family; transmembrane proteins with a single Ig-like domain expressed on the surface of T lymphocytes. Binding of either CD28 or CTLA-4 by CD80 or CD86 results in immune activation and the dampening of immune responses, respectively (6–8). Other members of the B7 family have been more recently described (5, 7). Among them, PD-L1 plays a role of growing importance. PD-L1 is constitutively present on monocytes and can be induced on activated T cells. PD-L1 binds to PD-1; this interaction results in the up-regulation of IL-10 production and the consequent reduction of T cells proliferation (9–12). In particular, PD-1 ligation inhibits PI3K activity, the downstream activation of Akt, and the induction of the cell survival factor Bcl-XL (13). The importance of this pathway in immune responses was further highlighted by data indicating that the PD-1/PD-L1 pathway plays a pivotal role in tolerizing and destroying self-Ags-specific cells, thus preventing autoimmunity (14).

In experimental autoimmune encephalomyelitis (EAE), a T cell-driven autoimmune disease that shares a number of clinical and pathological features with multiple sclerosis (MS), the interaction between PD-1 and PD-L1 regulates disease severity. In particular, PD-1 blockade results in an accelerated and more severe disease (15). The PD-1/PD-L1 pathway plays an important role in modulating immune functions in MS patients; monitoring and targeting these proteins could offer diagnostic and therapeutic advantages.
form of EAE (15), specific blockade of PD-1 results in EAE enhancement (16), ligation of PD-1 by PD-L1 attenuates T cell responses in EAE (17), and immunization of PD-L1−/− mice with myelin oligodendrocyte glycoprotein (MOG)35–55 or adoptive transfer of MOG35–55 specific T cells in such mice result in the development of a particularly severe form of EAE. In all these models, the exacerbation of EAE is likely driven both by a reduced production of Ag-specific IL-10 and a diminished rate of apoptosis of myelin-specific lymphocytes. With the notable exception of a report showing that B7-H1 is strongly up-regulated in human brain production of Ag-specific IL-10 and a diminished rate of apoptosis of B7-H3 expression in these patients.

**Materials and Methods**

One-hundred patients with MS diagnosed by clinical and laboratory parameters and followed by the Centro Sclerosi Multipla, Don Gnocchi Foundation in Milan, Italy, were included in the study. These patients were affected by relapsing remitting (RR)-MS with or without sequelae. The disease had been clinically stable in 38 patients for at least 6 months before the study period; these patients (median age, 37 years; range, 24–53 years; 25 females and 13 males) were classified as being affected by stable MS (SMS). The diagnosis of SMS was confirmed by brain and spinal cord magnetic resonance imaging (MRI) with gadolinium. MRI showed no arachnoiditis, enhancement of the periventricular white matter, or sulcal effacement.

**Patients and controls**

Eighty-two patients with RR-MS patients (median age, 40 years; range, 21–57 years; five females and five males) with median EDSS score 2.0; EDSS range, 1–5) who received glatiramer acetate (COPAXONE [COPA] from Sanofi-Aventis, 20 mg/ml s.c. daily), were analyzed as well. In all patients, therapy resulted in a significant reduction of relapse rate. All patients gave informed consent according to a protocol approved by the local ethics committee of the Don Gnocchi Foundation (Milan, Italy).

**Blood sample and CSF collection and cell separation**

Whole blood was collected by venepuncture in Vacutainer tubes containing EDTA (BD Biosciences). PBMC were separated on lymphocyte separation medium (Organon Teknika), washed twice in PBS, and the number of viable leukocytes was determined by trypsin blue exclusion. Ten milliliters of CSF were also obtained by lumbar puncture.

**Synthesis of the MBP peptides**

Thirty-one HLA I-restricted and seven HLA II-restricted promiscuous peptides partially overlapping and spanning the whole MBP were synthesized, using fluorenylmethoxycarbonyl chemistry. Peptides purity, as assayed by HPLC, was >70%, and their composition was verified by mass spectrometry. Lyophilized peptides were dissolved at 25 mg/ml in DMSO or sterile water to prepare peptide pools (10 μg/ml final concentration).

**Immunochemical analyses**

Analyses were performed using 50 μl of EDTA peripheral blood incubated for 30 min at 4°C with fluorochrome-labeled mAbs anti-CD4, -CD8, -CD14, -CD19, -CD80, -CD86, -PD-1, -PD-L1 (B7-H1), and -B7-H3. Erythrocyte lysis and cell fixation was performed using the Immuno-Prep EPICS kits and Q-prep Work Station (both from Beckman Coulter). CSF was processed at 4°C immediately after spinal tap. Cells were centrifuged after collection of 10 ml of CSF and stained using the same protocol to analyze CD4+ and CD8+ expressing PD-1 and CD14+ and CD19+ expressing PD-L1 in peripheral blood (see Immunochemical staining).

**Stimulation of PBMC for FACs analysis**

PBMC (5 × 10⁶) were incubated for 18 h in the presence of MBP peptide pools (10 μg/ml). Anti-CD28 mAb (clone 37407.111; R&D Systems) was added for 60 min at 4°C; cells were finally resuspended in 0.5 ml of FITC-labeled mAbs anti-CD4, -CD8, -CD14, -CD19, -CD80, -CD86, or -PD-1 mAbs for 30 min at 4°C with a rat anti-mouse anti-human PD-L1 (B7-H1) Ab (2.5 μg/ml) or with a hamster anti-mouse B7-H3 (2.5 μg/ml) (gifts from Dr. L. Chen, Johns Hopkins University School of Medicine, Baltimore, MD). After 30 min at 4°C, the cells were washed and further incubated for 30 min at 4°C with a rat anti-mouse IgG (H + L chain) Ab conjugated with FITC (1 μg/ml) or with a mouse anti-hamster IgG conjugated with PE (1 μg/ml). For the analysis of cytokine-secreting cells, PBMC were washed and fixed in reagent A solution (FIX & PERM cell permeabilization kits; Caltag Laboratories) for 10 min at room temperature in the dark. Cells were then resuspended in reagent B (FIX & PERM cell permeabilization kits), with cytokines-specific mAbs. After incubation cells were washed and fixed in 1% paraformaldehyde in PBS.

**Aptosis and phosphorylated AKT assays**

PBMC were stained with CD4+ and CD8-specific mAbs for 30 min at 4°C in the dark. For indirect immunofluorescence staining, PBMC were first incubated with a mouse anti-human PD-L1 (B7-H1) Ab (2.5 μg/ml) or with a hamster anti-mouse B7-H3 (2.5 μg/ml) (gifts from Dr. L. Chen, Johns Hopkins University School of Medicine, Baltimore, MD). After 30 min at 4°C, the cells were washed and further incubated for 30 min at 4°C with a rat anti-mouse IgG (H + L chain) Ab conjugated with FITC (1 μg/ml) or with a mouse anti-hamster IgG conjugated with PE (1 μg/ml). For the analysis of cytokine-secreting cells, PBMC were washed and fixed in reagent A solution (FIX & PERM cell permeabilization kits; Caltag Laboratories) for 10 min at room temperature in the dark. Cells were then resuspended in reagent B (FIX & PERM cell permeabilization kits), with cytokines-specific mAbs. After incubation cells were washed and fixed in 1% paraformaldehyde in PBS.
Monoclonal Abs

The following mAbs were used in this study: anti-human CD4 (clone 13B8.2; mouse IgG2a isotype), anti-human CD14 (clone 116; mouse IgG1 isotype), anti-human CD19 (clone J4.119; mouse IgG1 isotype) coupled to R-PE-Cyanine 5 Tandem (PE-Cy5; Caltag Laboratories); anti-human CD8 (clone SFCI21thy2D3; mouse IgG1 isotype) PE-Cyanin-7 (Beckman-Coulter); anti-human CD86 (clone BU63; mouse IgG1 isotype), anti-human CD80 (clone 3H5; mouse IgG1 isotype; Serotec) coupled to FITC; anti-human PD-L1 (gift from Dr. L. Chen) and anti-mouse IgG (H\(\text{H}\)/H11001 L\(\text{L}\) chain) coupled to FITC (eBioscience); anti-human-B7H3 (gift from Dr. L. Chen) coupled FITC anti-human PD-1 (clone MIH4) coupled to PE (mouse-IgG1 isotype; eBioscience); and anti-human annexin V coupled to FITC (mouse-IgG1 isotype; Beckman Coulter). The intracellular molecule detection mAb used was anti-human IL-10 (clone JES9D7; mouse IgG1 isotype; Caltag Laboratories) coupled to PE, anti-human IFN-\(\gamma\) (clone GZ4; mouse IgG1 isotype; Caltag Laboratories) coupled to PE, and anti-phospho-Akt (clone 193H12; Ser473) coupled to Alexa Fluor 488 (rabbit IgG isotype; Cell Signaling).

Cytometric analysis

Analyses were performed using a Beckman Coulter Cytomics FC-500 flow cytometer equipped with a single 15 mW argon ion laser operating at 488 nm, interfaced with CXP Software 2.1. One hundred thousand lymphocytes or 20,000 events were acquired and gated on CD4, CD8 (or CD14, CD19) expression, and side scatter properties. Green fluorescence from FITC (FL1) was collected through a 525-nm bandpass filter, orange-red fluorescence from R-PE (FL2) was collected through a 575-nm bandpass filter, deep-red fluorescence from PE-Cy5 (FL4) was collected through a 670-nm bandpass filter, and blue fluorescence from PE-Cy7 (FL5) was collected through a 770-nm bandpass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, FL4, and FL5. Samples were first run using isotype control or single fluorochrome-stained preparations for color compensation. Rainbow calibration particles (Spherotech), a mixture of several similarly sized particles with different fluorescence intensities, were used to standardize flow-cytometry results in samples obtained over time.

Table I. MFI of PD-1 on MBP-stimulated CD4\(^+\) and CD8\(^+\) T lymphocytes and of PD-L1 on CD14\(^+\) and CD19\(^+\) cells of SMS and AMS patients\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Median MFI (i.q. range)</th>
<th>p Value</th>
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<tr>
<td></td>
<td>SMS Patients</td>
<td>AMS Patients</td>
</tr>
<tr>
<td>CD4(^+)/PD1(^+)</td>
<td>16 (12–18)</td>
<td>12 (11–13)</td>
</tr>
<tr>
<td>CD8(^+)/PD1(^+)</td>
<td>13 (12–15)</td>
<td>11 (10–12)</td>
</tr>
<tr>
<td>CD14(^+)/PD-L1(^+)</td>
<td>26.5 (23.7–29.7)</td>
<td>5 (4–6.1)</td>
</tr>
<tr>
<td>CD19(^+)/PD-L1(^+)</td>
<td>11.9 (9.8–12.7)</td>
<td>13.4 (10.1–19.3)</td>
</tr>
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</table>

\(^a\) PD-1 MFI values were calculated on MFI-positive cells alone. i.q., Interquartile range; n.s., not significant.

FIGURE 1. MBP-stimulated CD4\(^+\) and CD8\(^+\) expressing PD-1. Representative results of MBP-stimulated PBMC of patients with either AMS or SMS are shown. Top panels, PD-1-expressing MBP-stimulated CD4\(^+\) T lymphocytes. Bottom panels, PD-1-expressing MBP-stimulated CD8\(^+\) T lymphocytes. In the upper right corners, the percentage of CD4\(^+\)/PD-1\(^+\) and CD8\(^+\)/PD-1\(^+\) T cells relative to the total number of lymphocytes are indicated. Summary results obtained in AMS and SMS patients are presented in A and B. The boxes stretch from the 25th to the 75th percentile; the lines across the boxes indicate the median values; the lines stretching from the boxes indicate extreme values. Statistical significance is shown.
Statistical analysis

Procedures were based on nonparametric analyses. Comparisons between the different groups were made using a two-tailed Mann-Whitney U test performed for independent samples. Possible relationships were evaluated using a Pearson’s correlation test. Statistical analysis was performed using the SPSS statistical package (SPSS Inc).

Results

CD28, CTLA4, B7.1 (CD80), and B7.2 (CD86) expression

No differences in CD28 expression were detected in any of the groups considered. A trend toward an increase of CD80-expressing CD14/H11001 and CD19/H11001 cells was seen in SMS compared with all other groups of patients, but these differences did not reach statistical significance (data not shown).

PD-1-expressing CD4+ and CD8+ T lymphocytes in peripheral blood and CSF

MBP-stimulated lymphocytes were analyzed in peripheral blood and fresh, unstimulated lymphocytes were studied in CSF. Results showed that MBP-stimulated and PD-1-expressing CD4+ and CD8+ T lymphocytes were significantly increased in peripheral blood of SMS compared with AMS patients (CD4+, p = 0.04; CD8+, p = 0.002) (Fig. 1). To determine whether these results were secondary to alterations in memory and naïve T lymphocytes populations, we characterized such populations, upon MBP stimulation, in 10 AMS and 10 SMS patients. Results indicated that the four main memory/naïve T lymphocyte populations identified by CCR7 and CD45RA surface proteins (CCR7+/CD45RA−, CCR7+/CD45RA+, CCR7−/CD45RA−, and CCR7−/CD45RA+) were comparable in both groups of patients (data not shown).

PD-1 mean fluorescence intensity (MFI), calculated on PD-1-expressing lymphocytes alone, was significantly increased in CD4+ and CD8+ (p = 0.03 in both cases) T cell of SMS patients (Table I). Analysis of PD-1 MFI in T lymphocytes obtained from the CSF, showed that liquoral CD4+ lymphocyte of SMS patients

<table>
<thead>
<tr>
<th></th>
<th>Median MFI (i.q. range)</th>
<th>p Value</th>
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<tbody>
<tr>
<td></td>
<td>SMS Patients</td>
<td>AMS Patients</td>
</tr>
<tr>
<td>CD4+/PD1+</td>
<td>21 (14–24)</td>
<td>17 (11–26)</td>
</tr>
<tr>
<td>CD8+/PD1+</td>
<td>15 (11–22)</td>
<td>13 (11–18)</td>
</tr>
<tr>
<td>CD14+/PD-L1+</td>
<td>23.8 (17.1–31.7)</td>
<td>3.5 (3–5)</td>
</tr>
<tr>
<td>CD19+/PD-L1+</td>
<td>11.1 (8.4–15.5)</td>
<td>4 (3–5)</td>
</tr>
</tbody>
</table>

*Results indicate values observed in cells from the cerebrospinal fluid. PD-1 MFI values were calculated on MFI-positive cells alone. i.q., Interquartile range; n.s., not significant.

FIGURE 2. MBP-stimulated CD14+ and CD19+ expressing PD-L1. Representative results of MBP-stimulated PBMC of patients with either AMS or SMS are shown. Top panels, MBP-stimulated PD-L1-expressing CD14+ cells. Bottom panels, MBP-stimulated PD-L1-expressing CD19+ cells. In the upper right corner of dot plots, the percentage of CD14+/PD-L1+ and CD19+/PD-L1+ are indicated. Summary results obtained in AMS and SMS patients are presented in A and B. The boxes stretch from the 25th to the 75th percentile; the lines across the boxes indicate the median values; the lines stretching from the boxes indicate extreme values. Statistical significance is shown.
expressed higher PD-1 MFI values as well, even though these differences did not reach statistical significance. These data are shown in Table II.

**PD-L1-expressing CD14⁺ and CD19⁺ cells**

PD-1 on T lymphocytes binds a specific receptor, PD-L1, on the surface of APCs. Because we detected an increased expression of PD-1 on T cells of SMS patients, we next analyzed PD-L1 expression and MFI in MBP-stimulated cells of all the patients enrolled in the study. Results showed that CD14⁺/PD-L1⁺ cells were increased in SMS in comparison to AMS patients, although the difference did not reach statistical significance (p = 0.066; Fig. 2). A similar trend was observed in CD19⁺ cells: the highest values of CD19⁺/PD-L1⁺ cells were present in SMS individuals; these differences were significant compared with the values observed in AMS patients (p = 0.009; Fig. 2). PD-L1 MFI was significantly increased in CD14⁺ cells of patients with a diagnosis of SMS, both in peripheral blood (p = 0.0007; Table I) and in CSF (p = 0.03; Table II).

**IL-10-secreting and PD-L1-expressing CD14⁺ and CD19⁺ cells**

The interaction between PD-1 and PD-L1 results in the preferential production of IL-10. We therefore analyzed IL-10-producing and PD-L1-expressing CD14⁺ and CD19⁺ cells in MBP-stimulated cultures of all the patients enrolled in the study; results showed that both types of cells were significantly increased in SMS compared with AMS patients (CD14⁺/PD-L1⁺/IL-10⁺ cells, p = 0.02; CD19⁺/PD-L1⁺/IL-10⁺ cells, p = 0.02). These data are shown in Fig. 3. We further analyzed IL-10 production in the CD14⁺/PD-L1⁺ and in the CD19⁺/PD-L1⁺ gated populations, i.e., we quantified the percentage of IL-10-producing cells in response to MBP within the pool of PD-L1-expressing CD14⁺ and CD19⁺ cells. Data indicated that the percentage of IL-10-producing cells was significantly increased in MBP-stimulated CD14⁺ and CD19⁺

### Table III. IL-10-production: percentage of PD-L1-expressing CD14⁺ and CD19⁺ cells that produce IL-10 in response to MBP stimulation

<table>
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<tr>
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<th>Median (i.q. range)</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>IL10⁺ (CD14⁺/PD-L1⁺)</td>
<td>20 (16–23)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL10⁺ (CD19⁺/PD-L1⁺)</td>
<td>50 (45–60)</td>
<td>&lt; 0.001</td>
</tr>
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</table>

AMS patients divided into patients with either stable multiple sclerosis (SMS) or acute multiple sclerosis (AMS). i.q., Interquantile range.

Percentage of cells.
cells of SMS compared with AMS patients (p < 0.001 in each population). These data are shown in Table III.

**MBP-stimulated PBMC apoptosis and proliferation**

Beside IL-10 production, the interaction between PD-1 and PD-L1 also results in cell apoptosis. We analyzed this parameter by evaluating 20 patients (10 AMS and 10 SMS) to determine whether: 1) PD-1 and annexin V would be coexpressed in MBP-stimulated CD4\(^+\) and CD8\(^+\) T lymphocytes; and 2) the percentage of MBP-stimulated PD-1 and annexin V coexpressing T lymphocytes would be different in AMS compared with SMS patients. Results indicated that both annexin V-expressing CD4\(^+\) and CD8\(^+\) populations of MBP-specific T lymphocytes were augmented in SMS compared with AMS individuals (p = 0.004 and p = 0.01, respectively; Fig. 4). These results were further confirmed by the observation that MBP-specific, pAkt-positive (proliferating) CD4\(^+\) and CD8\(^+\) lymphocytes were decreased in SMS compared with AMS patients (p = 0.03 and p = 0.01, respectively; Figure 5). Notably, coexpressing experiments showed that pAkt-expressing, MBP-stimulated T lymphocytes do not coexpress PD-1 (data not shown).

In cells of AMS, but not SMS, patients, preincubation with a blocking PD-1-specific Abs resulted in an augmented percentage of MBP-specific, pAkt-expressing (i.e., proliferating) CD4\(^+\) and CD8\(^+\) T lymphocytes. This effect was not seen upon the preincubation of cells with a PD-L1-specific Ab (Fig. 6).

**B7-H3 (CD276)-expressing CD14\(^+\) and CD19\(^+\) cells**

B7-H3 binds to a still undefined protein on the surface of T lymphocytes; this interaction results in the preferential production of IFN-\(\gamma\) and stimulates T cell activation and proliferation. B7-H3 expression was analyzed in MBP-stimulated cells of all the patients enrolled in the study. The highest values of B7-H3-expressing cells were observed in AMS individuals but these differences did not reach statistical differences (data not shown).

**IFN-\(\gamma\)-secreting and B7-H3-expressing CD14\(^+\) and CD19\(^+\) cells**

IFN-\(\gamma\)-producing and B7-H3-expressing CD19\(^+\) and CD14\(^+\) cells were analyzed in MBP-stimulated cultures; both cell populations were comparable in all groups of patients examined (data not shown).

**PD-L1-IL-10/B7-H3-IFN-\(\gamma\) ratio in CD14\(^+\) and CD19\(^+\) cells**

The ratio of PD-L1/IL-10 to B7-H3/IFN-\(\gamma\) was calculated in CD14\(^+\) and CD19\(^+\) cells in all patients. Results indicated that in
both cell populations this ratio was reduced in AMS compared with SMS individuals. The differences were statistically significant in CD19⁺ cells (p = 0.021).

Effect of therapy on the expression of costimulatory molecules
To analyze whether therapy-induced control on disease progression induces changes in the PD-1/PD-L1 circuit, IFN- and COPA-treated patients were included in the study. All patients responded to therapy as established by clinical parameters. PD-1-expressing cells were slightly increased in the peripheral blood of patients undergoing both therapies compared with AMS patients; these differences were not statistically significant (data not shown). In contrast, MBP-stimulated CD19⁺ CD4⁺ and CD8⁺ T lymphocytes expressing PD-L1 were significantly reduced in IFN-treated patients as compared with SMS patients (p = 0.026). Finally, MBP-stimulated CD19⁺ IL-10⁺ PD-L1⁺ cells were significantly reduced in COPA-treated patients as compared with SMS patients (p = 0.032; Table IV). These results argue against the possibility that IFN- and COPA-induced disease remission is mediated by the modulation of PD-L1 expression.

Discussion
We analyzed the expression of the B7 family of costimulatory proteins in patients affected by different forms of MS; we also verified that diverse patterns of expression would have resulted in different cytokine profiles and in a diverse rate of apoptosis and proliferation of Ag-specific cells in these patients. Our results indicate that, whereas the expression of CD28, CD80, and CD86 was only marginally different in MS patients with diverse patterns of disease, a tight correlation exists between quiescent phases of MS and a significantly up-regulated expression of the PD-1/PD-L1 pair of costimulatory molecules. Notably, this resulted in a higher production of the anti-inflammatory cytokine IL-10 in MBP-stimulated cells, as well as in a higher rate of apoptosis and a lower proliferation rate of MBP-specific CD4⁺ and CD8⁺ T lymphocytes. In contrast with these results, B7-H3 expression was not different in AMS or SMS; accordingly, MBP-stimulated IFN-γ-producing and B7-H3-expressing cells were comparable in both groups of patients, even though the PD-1/PD-L1/B7-H3-IFN-γ ratio was significantly lower in AMS compared with SMS patients. Finally, successful therapy with two commonly used immune-modulators, COPA and IFN-α/β, resulted in a down-regulation of PD-L1-expressing cells and of IL-10-producing/PD-L1-expressing cells, suggesting that the disease remission induced by these compounds is not associated with the modulation of the PD-1/PD-L1 pathway.

The interaction between PD-1 and PD-L1 regulates the reciprocal balance between T cell tolerance and activation (13, 14, 31). Recent data show that the ligation of PD-L1 on the surface of
dendritic cells by naive T lymphocytes limits effector cell responses and prevents the triggering of immune-mediated tissue damage (13, 14, 31). This is achieved through two major and complementary mechanisms: the generation of IL-10, a powerful inflammation-dampening cytokine; and the limitation of the proliferation of Ag-specific cells, possibly via the triggering of apoptosis of such cells. In fact, PD-1 is abundantly expressed in diseases associated with increased IL-10 generation such as lung, ovary, and colon carcinoma (32, 33) and HIV infection (34, 35). In contrast, PD-1/PD-L1 interactions modulate positive and negative selection in the thymus (36, 37) and protect immune privileged sites such as the placenta (38, 39) and the eye from immune responses (40, 41).

Experimental data indicate a pivotal role for alterations in the PD-1/PD-L1 pathway in a number of inflammatory conditions, including diabetes (42) and inflammatory bowel diseases (43). In the case of MS, studies performed in the EAE model in mice showed that PD-1- and PD-L1-expressing cells infiltrate the brain of mice with EAE (44). Further data showed that the use of anti-PD-1 during the development of EAE accelerates the development of disease and results in an augmentation of MOG-specific, IFN-γ-producing T lymphocytes. Other results obtained in the EAE model reinforce the importance of the PD-1/PD-L1 pathway in the EAE model. To summarize: 1) severe EAE develops following immunization of PD-L1−/− mice with MOG or following the adoptive transfer of MOG35–55-specific cells (45); 2) PD-L1−/− B57BL/6 mice develop a much more severe form of EAE following adoptive transfer of MOG35–55-specific lymphocytes compared with the wild-type controls (46); 3) the 129Sv strain of mice, a strain resistant to EAE, can be rendered susceptible to disease by PD-L1 deficiency (46); and 4) the regulatory contributions of B lymphocytes during EAE are linked to their ability to produce IL-10 (47–50). Results indicating that the up-regulation of the PD-1/PD-L1 pathway is associated with quiescent phases of disease offer support to the concept that this pathway plays a pivotal role in this family of diseases. Notably, our results indicate that PD-L1-expressing and IL-10-producing B lymphocytes are increased in SMS patients, confirming an immune regulatory role for these cells in MS.

It has been shown that, beside PD-L1, PD-1 can bind a second protein, PD-L2, on the surface on non-T cells (44). We did not

### Table IV. MBP-stimulated PD-L1-expressing and PD-L1-expressing/IL-10-producing CD14+ and CD19+ cells of patients with MS undergoing successful therapy with either IFN-α/β (IFNs) or COPA

<table>
<thead>
<tr>
<th>MBP-stimulated PBMC Median (i.q. range)</th>
<th>IFNs</th>
<th>COPA</th>
<th>SMS</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+/PD-L1+ (%)</td>
<td>7.9 (1.0–17.9)</td>
<td>2.2 (0.9–5.2)</td>
<td>2.8 (1.9–4.6)</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD19+/PD-L1+ (%)</td>
<td>0.5 (0.3–1.5)</td>
<td>2.2 (0.5–2.9)</td>
<td>2.9 (1.7–4.8)</td>
<td>0.026b</td>
</tr>
<tr>
<td>CD14+/PD-L1+/*IL-10+ (%)</td>
<td>1.1 (0.9–3.6)</td>
<td>0.3 (0.1–0.9)</td>
<td>1.7 (0.6–4.7)</td>
<td>0.032c</td>
</tr>
<tr>
<td>CD19+/PD-L1+/*IL-10+ (%)</td>
<td>0.4 (0.04–0.7)</td>
<td>0.8 (0.4–1.6)</td>
<td>1.3 (0.7–2.4)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

a Cell percentages are shown i.q., Interquantile range. n.s., not significant.
b vs. IFN-α/β.
c vs. COPA.
analyze the expression of PD-L2 in our patients because previous data indicated that immunization of mice with MOG \textsuperscript{35–55} provokes a progressive increase of PD-1 and PD-L1, but not PD-L2, within the CNS of EAE mice (15). Additional results show that the PD-1/PD-L1, but not the PD-1/PD-L2, interaction regulates the severity of EAE (17, 45). Although these data were subsequently partially refuted by other results showing that PD-L2 can play a role in EAE in some mouse strains (16), we felt supported in our decision by the observation that the expression of PD-L2 is much more restricted than that of PD-L1. Thus, whereas PD-L1 is constitutively expressed on a wide range of hematopoietic and nonhematopoietic cells (46, 51, 52), PD-L2 is only inducible expressed in dendritic cells, macrophages, and mast-cells (46, 51–53).

Incubation of PBMC of AMS patients with PD-1-specific neutralizing Abs resulted in the increased proliferation of MBP-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes. Notably, this effect was not seen when cells were pretreated with a PD-L1-specific Ab and was not observed in cells of SMS patients. The different biological behavior observed in ASM compared with SMS cells could indicate that in SMS individuals, MBP-specific immune reactivity is kept under tight control by multiple mechanisms, as in the in vitro modification of a single parameter is not capable of altering the overall balance of MBP-specific immune responses. In particular, in SMS patients, PD-L1 could bind B7.1 (13), a molecule that is up-regulated in such patients; this interaction would result in an inhibitory signal. In contrast, these responses are up-regulated in AMS, as the blocking of PD-1 leads to a higher proliferation of MBP-specific cells, reflecting an augmented degree of Ag-specific immune reactivity. In these patients, the reduced expression of all of the inhibitory molecules analyzed (PD-1, PD-L1, and B7.1) could justify the observation that the blockade of any of these receptors is sufficient to alter a delicate balance between activatory and inhibitory immune pathways. These results indicate that impeding the PD-1/PD-L1 interaction results in the dampering of an important immunoregulatory loop.

Blocking the PD-1/PD-L1 pathway was shown to augment the proliferation of HIV-specific CD8 T lymphocytes in chronically HIV-infected individuals (54). Our results confirm these findings and extend them to another human disease condition. Notably, whereas the ultimate goal of therapy in HIV infection is to reinforce Ag-specific immune responses, in MS patients such immune modulation would be potentially harmful. The lack of effect seen upon ligation of PD-L1 could be secondary to the possibility that, in the absence of available PD-L1, PD-1 will ligate PD-L2, resulting in a similar reduction of MBP-specific proliferation. These in vitro results indicate a possible limited role for PD-L2 in MS are in agreement with data indicating that PD-L2 plays only an ancillary role in regulating the severity of EAE in most mouse strains (15, 17, 18).

Whereas the PD-1/PD-L1 circuit was differently modulated in AMS and SMS patients, as both the percentages of immune cells expressing these molecules and the MFI were augmented in patients in whom disease was in a quiescent phase, changes in the expression and the MFI of B7-H3 were only marginal. Triggering of B7-H3 results in cell proliferation and in the production of IFN-γ, and thus in the elicitation of a proinflammatory environment (46), a situation that is harmful in MS. It is nevertheless interesting to note that, even though B7-H3 was only marginally different in AMS and SMS patients, the PD-L1/γ\textsuperscript{IL-10}/B7-H3/IFN-γ ratio was significantly reduced in AMS individuals.

These results suggest that the role played by the B7-H3 circuit in the modulation of disease activity in MS patients is subordinate to that of the PD-1/PD-L1 circuit, and that spontaneously achieved disease control in MS is driven by increments of IL-10 production, rather than by reductions of IFN-γ.

A recent paper showed that IFNβ treatment of MS is associated with the up-regulation of both PD-1 and PD-L1 expression on immune cells (55). Our results, obtained in patients who were undergoing therapy with either IFNα/β or COPA, did not confirm such data, as we did not observe changes in PD-1 expression and saw a decrease, rather than an increase, of PD-L1 expression in our patients. The discrepancy could at least partially be explained by the fact that, whereas Wiesemann et al. (55) analyzed unstimulated cells, we measured the expression of B7 costimulatory molecules on MBP-stimulated cells. We have recently shown that disease remission in MS is correlated with increased percentages of circulating MBP-specific naïve regulatory T lymphocytes (56); our results seem to indicate that such cells, and not the PD-1/PD-L1 circuit, mediate immune-therapy-mediated disease remission in MS.

In summary, data in this study indicate an important role for the PD-1/PD-L1 costimulatory pathway in modulating disease activity in MS. Thus, increased expression of PD-1 and PD-L1 and the resulting higher IL-10 production, lower proliferation, and increased apoptosis of MBP-specific cells is associated with disease remission in MS patients. These data confirm the importance of this pathway in human pathology; PD-1/PD-L1-directed therapeutic strategies could be envisioned as a way to design immunosuppressive approaches to MS and, possibly, other inflammatory diseases.

Disclosures
The authors have no financial conflict of interest.

References


