HLA-B7–Restricted EBV-Specific CD8+ T Cells Are Dysregulated in Multiple Sclerosis

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*J Immunol* 2012; 188:4671-4680; Prepublished online 28 March 2012; doi: 10.4049/jimmunol.1103100

http://www.jimmunol.org/content/188/9/4671

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/03/28/jimmunol.1103100.DC1

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It was hypothesized that the EBV-specific CD8⁺ T cell response may be dysregulated in multiple sclerosis (MS) patients, possibly leading to a suboptimal control of this virus. To examine the CD8⁺ T cell response in greater detail, we analyzed the HLA-A2-, HLA-B7-, and HLA-B8-restricted EBV- and CMV-specific CD8⁺ T cell responses in a high number of MS patients and control subjects using tetramers. Content in cytolytic granules, as well as cytotoxic activity, of EBV- and CMV-specific CD8⁺ T cells was assessed. We found that MS patients had a lower or a higher prevalence of HLA-A2 and HLA-B7, respectively. Using HLA class I tetramers in HLA-B7⁺ MS patients, there was a higher prevalence of MS patients with HLA-B*0702/EBV RPP-specific CD8⁺ T cells ex vivo. However, the magnitude of the HLA-B*0702/EBV RPP-specific and HLA-B*0702/CMV TPR-specific CD8⁺ T cell response (i.e., the percentage of tetramer⁺ CD8⁺ T cells in a study subject harboring CD8⁺ T cells specific for the given epitope) was lower in MS patients. No differences were found using other tetramers. After stimulation with the HLA-B*0702/EBV RPP peptide, the production of IL-2, perforin, and granzyme B and the cytotoxicity of HLA-B*0702/EBV RPP-specific CD8⁺ T cells were decreased. Altogether, our findings suggest that the HLA-B*0702-restricted viral (in particular the EBV one)-specific CD8⁺ T cell response is dysregulated in MS patients. This observation is particularly interesting knowing that the HLA-B7 allele is more frequently expressed in MS patients and considering that EBV is associated with MS.

The online version of this article contains supplemental material.

Received for publication October 27, 2011. Accepted for publication March 3, 2012.

This work was supported by grants from the Swiss National Foundation (PPOOB-106716, PPOOF3-124893), the Swiss Society for Multiple Sclerosis, and the Biaggi Foundation (to R.A.D.P.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: CIS, clinically isolated syndrome; CTL, cytotoxic CD8⁺ T lymphocyte; Grm, granzyme; MFI, mean fluorescence intensity; MS, multiple sclerosis; NIND, noninflammatory neurologic disease; PD-1, programmed cell death 1; rHL-2, recombinant human IL-2.

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Published online December 20, 2011.

E nvironmental and genetic factors are both thought to be involved in the pathogenesis of multiple sclerosis (MS) (1, 2). Among the latter, HLA class I alleles were shown to be associated with MS either in a protective (HLA-A2) or nonprotective (HLA-A3, HLA-B7) manner (3, 4). With regard to the environmental factors, sero-epidemiological studies revealed that the γ-herpesvirus EBV was associated with MS (5, 6). We and other investigators demonstrated that EBV-specific cellular immune responses are increased not only in the blood (7–10) but also in the cerebrospinal fluid of MS patients (11). Interestingly, we showed that the increase in EBV-specific CD8⁺ T cell response was maximal at disease onset but decreased with disease duration (9). Others investigators found a decreased reactivity to EBV-infected B cells in MS patients, which was not correlated with disease duration. However, in the latter study, clinically isolated syndrome (CIS) patients were not included (12).
(23). With regard to EBV-infected B cells, their killing is mediated by the perforin/granzyme pathway rather than by the Fas/Fas ligand pathway (24).

We previously demonstrated that MS patients exhibited a strong EBV-specific CD8+ T cell response in the early course of MS, a response that decreased significantly with the progression of the disease (9). In this study, we sought to determine whether EBV-specific CD8+ T cells of MS patients were intrinsically dysregulated.

Materials and Methods

Patients

We performed HLA class I (A and B) typing on 439 consecutive subjects, including 221 MS patients and 218 control subjects. The MS patient group included 43 patients with CIS, 123 patients with remitting-relapsing MS, 28 patients with secondary-progressive MS, and 27 patients with primary-progressive MS. Storage and analysis of clinical data from patients with MS were performed using iMed Software (Merck Serono, Geneva, Switzerland). At the time of enrollment, the diagnosis of MS was made using the 2005 revised McDonald criteria (25). The control subject group included 74 patients with other inflammatory neurologic diseases, 100 patients with noninflammatory neurologic diseases (NIND), and 44 healthy subjects. This study was accepted by the review board of the University Hospital of Lausanne, and all subjects gave their written informed consent.

None of the study subjects had received corticosteroids within the 3 mo prior to enrollment. MS patients were considered relapsing if a relapse had started <4 wk prior to the blood draw. Thirty-four patients were relapsing at the time of the assay.

HLA-A2 was shown to be a protective allele in MS, because it is less common in MS patients than in the general population (26), in contrast to HLA-B7, which is more prevalent in MS patients (27). Therefore, we were particularly interested in studying the virus-specific CD8+ T cell response restricted by these two HLA class I alleles. HLA-B8 has not been associated with MS and was used as a “control” allele. Thus, the studies described hereafter were performed on MS patients or control subjects who harbored at least one of these three HLA class I alleles.

Because the prevalence of HLA-A*02, HLA-B*07, and HLA-B*08 haplotypes, other than the common HLA-A*0201, HLA-B*0702, or HLA-B*0801 haplotype, is very low in the Swiss population (28), all of the study subjects had a two-digit HLA typing.

Serologies

To determine the EBV and CMV status (the latter virus being used as a control (9, 11)), EBV and CMV serologies were performed in those study subjects who had at least one of the three HLA class I alleles of interest. For EBV, plasma IgG titers were determined for EA, VCA, and EBNA-1, with the following Abs: CD3–allophycocyanin–Cy7, CD8-Pacific blue, IFN-γ–FITC, IL-2–PE (all from Becton Dickinson). An intra-vivo tetramer staining was considered as positive if >0.05% of CD3+CD8+ T cells were tetramer+ and if the tetramer+ cells were clearly demarcated from the other cells.

Content in IFN-γ, TNF-α, and IL-2 in EBV-specific CD8+ T cells

To determine which patients had a virus epitope-specific CD8+ T cell response, PBMC of the patients harboring at least one of the three HLA class I of interest and with positive serology for EBV and/or CMV were stained with the tetrarmers listed in Table I. Fresh PBMC were stained for 30 min at 4°C with the PE-coupled tetrarmers. CD3–PerCP-Cy5.5 and CD8-allophycocyanin Abs (both from Becton Dickinson, Franklin Lakes, NJ) were added for 20 min at 4°C. Cells were washed, and data were acquired on a FACScan flow cytometer (Becton Dickinson). An ex vivo tetramer staining was considered as positive if >0.05% of CD3+CD8+ T cells were tetramer+ and the tetramer+ cells were clearly demarcated from the other cells.

Ex vivo analysis of cytotoxic granule content and differentiation markers in EBV- and CMV-specific CD8+ T cells

To examine the ex vivo content in perforin, GrmA, GrmB, and GrmK of virus epitope-specific CD8+ T cells, 1–2 million PBMC were incubated with the EBV or CMV tetrarmers at 4°C for 30 min, permeabilized with Cytofix/Cytoperm (Becton Dickinson), and stained at room temperature for 20 min with GrmK-FITC (Santa Cruz Biotechnology, Santa Cruz, CA). GrmA-Pacific blue, perforin-allophycocyanin (both from BioLegend, San

EBV DNA

Detection of EBV DNA was performed in plasma and PBMC using real-time PCR (10). DNA was extracted from plasma and PBMC with the QiAamp DNA Mini Kit (Qiagen, Basel, Switzerland). The set of primers and probe were located in the BamHI W fragment of EBV. The forward primer was 5′-GGACCACCTGGCCCCCTGTA-3′, the reverse primer was 5′-TTTGTGGATCTCCTGG-3′, and the probe was 6-FAM-5′-TCCGT-CACGATTTCTGGCCTACATA-3′-TAMRA. The real-time PCR reaction was performed as previously described (29). Positive controls for EBV DNA detection were B-lymphoblastoid cell lines derived from an MS patient in our laboratory. All preparatory PCR steps, including DNA extraction, were done in a separate room isolated from any post-PCR samples to prevent contamination. Because of a shortage of PBMC in 10 study subjects, the interval between the real-time PCR assay and the cellular assays was 0.02 ± 0.9 y for MS patients and 0 ± 0.1 y for control subjects.

PBMC were stained for 18 h with one of the five peptides used in this study (Table I) in complete medium containing 1 μ/ml GolgiPlug (Becton Dickinson) and 0.5 μg/ml anti-CD28 Ab (Becton Dickinson). An intracellular cytokine staining assay was performed, as previously described (9), with the following Abs: CD3–allophycocyanin–Cy7, CD8-Pacific blue, IFN-γ–allophycocyanin, TNF-α–FITC, IL-2–PE (all from Becton Dickinson), and CD4-EDC (Beckman Coulter). Dead cells were excluded using the Aqua LIVE/DEAD stain kit (Invitrogen, Basel, Switzerland). Data were acquired on a LSRII flow cytometer (Becton Dickinson). PBMC stimulated with complete medium alone were used to determine the background. The background values never exceeded 0.1% of CD3+CD8+ T cells, and they were subtracted from the stimulated sample values before analysis.

Table I. EBV- and CMV-specific tetramers used in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>HLA restriction</th>
<th>Protein</th>
<th>Position</th>
<th>Peptide</th>
<th>Tetramer Core Facility, Emory University, Atlanta, GA</th>
<th>References</th>
<th>Obtained From</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>HLA-A*0201</td>
<td>BMLF1 (lytic)</td>
<td>259–267</td>
<td>GLCLTLVAML</td>
<td>HLA-A2/EBV&lt;sub&gt;veclc&lt;/sub&gt;</td>
<td>Bhardawaj et al. (52)</td>
<td>Beckman Coulter, Fullerton, CA</td>
</tr>
<tr>
<td>EBV</td>
<td>HLA-B*0702</td>
<td>EBNA-3a (latent)</td>
<td>379–387</td>
<td>RRPFIGIRL</td>
<td>HLA-B7/EBV&lt;sub&gt;vekpp&lt;/sub&gt;</td>
<td>Hill et al. (53)</td>
<td>Champagne et al. (31)</td>
</tr>
<tr>
<td>EBV</td>
<td>HLA-B*0801</td>
<td>BZLF1 (lytic)</td>
<td>190–197</td>
<td>RAKFKQQL</td>
<td>HLA-B8/EBV&lt;sub&gt;vekak&lt;/sub&gt;</td>
<td>Bogdian et al. (54)</td>
<td>National Institutes of Health Tumor Immunology Correlation Program, Emory University, Atlanta, GA</td>
</tr>
<tr>
<td>CMV</td>
<td>HLA-A*0201</td>
<td>pp65</td>
<td>495–503</td>
<td>NLVPVMAT</td>
<td>HLA-A2/CMV&lt;sub&gt;veclv&lt;/sub&gt;</td>
<td>Diamond et al. (55)</td>
<td>Beckman Coulter, Fullerton, CA</td>
</tr>
<tr>
<td>CMV</td>
<td>HLA-B*0702</td>
<td>pp65</td>
<td>417–426</td>
<td>TRPRVTGGGAM</td>
<td>HLA-B7/CMV&lt;sub&gt;vefpr&lt;/sub&gt;</td>
<td>Wills et al. (56)</td>
<td>Champagne et al. (31)</td>
</tr>
</tbody>
</table>
Diego, CA). GrmB-Alexa Fluor 700, CCR7–PE–Cy7, CD8–PerCP–Cy5.5 (all from Becton Dickinson), or CD57–PE–Cy5 (Abcam, Cambridge, U.K.) (23). For programmed cell death 1 (PD-1) staining, PBMC were incubated with the tetramer, as described above, and stained with CD3–ECD (Beckman Coulter), CD8–allophycocyanin–H7 (Becton Dickinson), and PD-1–FITC (Becton Dickinson). Cells were acquired on a LSRII flow cytometer (Becton Dickinson).

Analysis of cytotoxic granule content in EBV- and CMV-specific CD8+ T cells after short-term cultures

To examine the change in the cytotoxic granule content of the virus-specific CD8+ T cell response upon antigenic challenge, 2–4 million PBMC were stimulated with peptides, according to the HLA class I typing of studied subjects. Peptides were added at 1 μg/ml, and PBMC were cultured for 7 d in RPMI 1640 supplemented with 8% human serum (Institut de Biotechnologies Jacques Boy, Reims, France) in the presence or absence of 20 U/ml recombinant human IL-2 (rhIL-2; Roche Diagnostics, Rotkreuz, Switzerland). When rhIL-2 was added, it was done 48 h after the start of the culture. After 7 d, cultured PBMC were washed and stained with the corresponding tetramers, as described above. Data were acquired on an LSRII flow cytometer (Becton Dickinson).

Functional CFSE CTL assay

To examine the virus peptide epitope-specific CTL functional response upon antigenic challenge, 2–4 millions PBMC were stimulated with the EBV- or CMV-specific HLA-A*0702–restricted peptides. After 7 d, cultured PBMC were used as effector cells in a functional CFSE CTL assay. Presence of EBV- and CMV-specific CTL was assessed using the functional CFSE-based CTL assay, as previously described (11). Briefly, target cells were prepared by staining autologous PBMC with 0.25 μM CFSE (Invitrogen) and loading them for 3 h with the relevant Ag at 1 μg/ml (EBV or CMV HLA-A*0702–restricted peptides) or an irrelevant HIV Clade C 15-mer GagHPVHAGPIAPGQMRE peptide. After incubation, cells were washed and used as targets in the CTL assay. Four thousand target cells/well were incubated in U-bottom 96-well plates for 18 h in a total volume of 200 μl/well. Importantly, the assay was normalized in terms of tetramer effectors, whereas the percentage of tetramer+ cells after culture was assessed, and the final effector:target ratio was 0.1, 0.1/1, 0.25/1, 0.5/1, 1/1, and 2:1. The wells were harvested, and a constant number of allophycocyanin-fluorescent beads (Becton Dickinson) was added just before acquisition on a FACScalibur flow cytometer (Becton Dickinson) to normalize the number of CFSE-bright cells to the number of beads in each tube. The specific lysis was calculated according to the following formula: percentage specific lysis = 1 – (number of CFSE-bright cells/number of beads for a particular E:T ratio)/(number of CFSE-bright cells/number of beads at 0:1 ratio) × 100, where the 0:1 ratio represents no effectors cells. The background lysis in the presence of the irrelevant HIV peptide at different E:T ratios was subtracted from the EBV- or CMV-specific lysis results.

Statistical analysis

Flow cytometry data were acquired using FlowJo (Tree Star, Ashland, OR) and SPICE (developed by Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health) software programs. Analysis and presentation of distributions were performed using SPICE version 5.1, downloaded at http://exon.niaid.nih.gov/spice (30).

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA). The differences among the groups of study subjects were tested using the Kruskal–Wallis test for nonnormally distributed variables. The difference between two groups was tested using the nonparametric Mann–Whitney ranked test, whereas paired samples were tested with the nonparametric Wilcoxon-ranked test. Analyses of frequencies were performed using the Fisher exact test. A p value < 0.05 was considered significant.

Results

HLA-B7 is more prevalent in MS patients

Of the 439 consecutively enrolled study subjects, 307 (70%) were HLA-A*02+, HLA-B*07+, and/or HLA-B*08+. As found by other investigators (26, 27), the prevalence of HLA-A2 was lower in MS patients (33%) compared with control patients/subjects (52%, p = 0.0002), whereas the prevalence of HLA-B7 was higher in MS patients (32%) compared with control subjects (21%, p = 0.013).

The prevalence of HLA-B8 was identical in both MS patients (18%) and controls (18%; Fig. 1).

Subsequent experiments were not performed in 91 of the 307 HLA-compatible study subjects because of a lack of available PBMC or loss to follow-up. All 220 remaining study subjects were infected by EBV, except for 1 NIND control subject. Ninety-nine of the 220 (45%) were infected by CMV, including the NIND subject who was negative for EBV. Clinical and paraclinical data for these 220 study subjects are displayed in Table II.

Higher prevalence of HLA-B7/EBV-specific CD8+ T cells in MS patients

We then assessed the prevalence of EBV- and CMV-specific CD8+ T cells ex vivo using HLA-A*0201, HLA-B*0702, and HLA-B*0801/viral peptide epitope tetramers in the 220 study subjects (Table II). A positive tetramer staining specific for at least one viral epitope was found in 155/220 study subjects (70%). Interestingly, we found that the prevalence of HLA-B7/EBV-specific CD8+ T cells was higher in HLA-B7+ MS patients (43/59 [73%]) than in HLA-B7+ control subjects (19/39 [49%], p = 0.019; Fig. 2A). In contrast, there was no significant difference between these two categories of study subjects in terms of the prevalence of CD8+ T cells specific for the four other viral epitopes (Fig. 2).

Contrasting with their higher prevalence in MS patients compared with control subjects, the magnitude of HLA-B7/EBV-specific CD8+ T cells (i.e., the percentage of tetramer+ CD8+ T cells in a study subject harboring CD8+ T cells specific for a given epitope) tended to be lower (p = 0.07; Fig. 2A). The magnitude of HLA-B7/CMV-specific tetramer cells was significantly lower in MS patients (p = 0.004; Fig. 2B). In contrast, there was no difference between MS patients and control subjects in terms of the magnitude of tetramer staining for HLA-A2/EBV, HLA-A2/CMV, or HLA-B8/EBV (Fig. 2). Of note, nine MS

FIGURE 1. Prevalence of HLA-A2, HLA-B7, and HLA-B8 in our cohort of 439 patients. The percentages indicate the number of study subjects carrying the HLA allele of interest compared with the total amount of subjects tested. *p < 0.05. **p < 0.001. CTRL, Control subjects including other neurologic diseases patients and healthy subjects; MS, MS patients.
patients were on IFN-β treatment, including six who were relapsing, suggesting that the immunomodulatory effect of IFN-β was overruled by the inflammation related to the relapse and, thus, reasonably ruling out a significant role for IFN-β in our results.

**Production of IFN-γ and IL-2 is decreased in MS patients after HLA-B7/EBV<sub>RPP</sub> peptide stimulation**

Having found that the presentation of EBV<sub>RPP</sub> and CMV<sub>TPR</sub> immunodominant epitopes by the HLA-B7 allele induced a different CD8<sup>+</sup> T cell response between MS patients and control subjects, we looked at the effector functions of these viral peptide epitope-specific CD8<sup>+</sup> T cells. Specifically, we determined the ex vivo production of IFN-γ, TNF-α, and IL-2 by the five viral peptide epitope-specific CD8<sup>+</sup> T cells and found no differences between MS patients and control subjects.

However, we were also interested in determining the amount of cytokine secreted by viral peptide epitope-specific CD8<sup>+</sup> T cells. To this end, we examined the mean fluorescence intensity (MFI), which reflects the amount of cytokine produced by a single cell. We found that HLA-B7–restricted EBV<sub>RPP</sub>–specific CD8<sup>+</sup> T cells of MS patients exhibited a lower MFI for IFN-γ and IL-2 than did those of control subjects (p = 0.015 and p = 0.0003, respectively, Fig. 3A). In contrast, there was no difference in MFI between these two categories for both HLA-A2–restricted EBV<sub>GLC</sub>–specific CD8<sup>+</sup> T cells and HLA-B8–restricted EBV<sub>RAK</sub>–specific CD8<sup>+</sup> T cells (Fig. 3A).

For the CMV–specific CD8<sup>+</sup> T cell responses, there was no difference in the MFI for HLA-A2–restricted CMV<sub>NLV</sub>–specific CD8<sup>+</sup> T cells for all three cytokines, whereas there was a trend for an increased secretion of IL-2 in HLA-B7–restricted CMV<sub>TPR</sub>–specific CD8<sup>+</sup> T cells in MS patients (p = 0.053; Fig. 3B).

**The content in cytolytic granules of EBV- and CMV-specific CD8<sup>+</sup> T cells is normal in MS patients**

To pursue our detailed analysis of this seemingly dysregulated virus-specific CD8<sup>+</sup> T cell response, we investigated the ex vivo content in cytolytic granules (perforin, GrmB, GrmA, and GrmK) of HLA-A2, HLA-B7, or HLA-B8 tetramer<sup>+</sup>CD8<sup>+</sup> T cells, regardless of the category of study subjects (23). To perform an in-depth characterization, a sufficiently high number of tetramer<sup>+</sup>CD8<sup>+</sup> T cells is needed. Thus, we included 77 subjects who had ≥0.05% CD3<sup>+</sup>CD8<sup>+</sup> tetramer<sup>+</sup> cells ex vivo out of 152 tetramer<sup>+</sup> subjects.

We found no major difference in the total content in perforin, GrmB, GrmA, or GrmK in EBV- or CMV-specific CD8<sup>+</sup> T cells between MS patients and control subjects (Supplemental Fig. 1B, 1C).

Furthermore, we analyzed the 16 populations of tetramer<sup>+</sup> cells using SPICE software, as characterized by the 16 possible combinations of cytolytic granules. As previously shown in our laboratory, the distribution of perforin, GrmB, GrmA, and GrmK differed markedly between EBV- and CMV-specific CD8<sup>+</sup> T cells, regardless of the category of study subjects (23); however, we did not find any difference between MS and control subjects (Supplemental Fig. 2A, 2B).

**Virus-specific CD8<sup>+</sup> T cells of MS patients have similar expression of CCR7, CD57, and PD-1 markers compared with control subjects**

To determine whether the differences between MS patients and control subjects in terms of HLA-B7–restricted viral-specific CD8<sup>+</sup> T cell responses were due to differences in their pattern of differentiation, we analyzed the cell surface expression of CCR7,
a marker of cell differentiation (31); CD57, a marker of senescence (32); and PD-1, a marker of CD8+ T cell proliferation and viral exposure (33). As described previously (34), we found that tetramer+ cells were mostly CCR7- for both viruses (97% for EBV and 99% for CMV; Supplemental Fig. 3A), indicating that they were either effector memory or effector CD8+ T cells (35). However, no difference was seen between MS and control subjects for either EBV or CMV. Similarly, there was no difference in the expression of CD57, with the exception of HLA-B8/EBVRAK CD8+ T cells of MS patients, which expressed less CD57 (Supplemental Fig. 3B). Finally, we found no difference in the expression of PD-1 between the two groups (Supplemental Fig. 3C). These data show that the decreased magnitude of the HLA-B7–restricted viral-specific CD8+ T cell response that we observed in MS patients cannot be attributed to exhaustion or lack of differentiation of these cells.

Upon Ag stimulation in short-term culture, the cytotoxic phenotype of HLA-B7/EBVRPP–specific CD8+ T cells of MS patients is deficient

To further characterize the viral-specific CD8+ T cell response restricted by the different HLA class I, we set up a 1-wk stimulation. We found that HLA-B7/EBVRPP–specific CD8+ T cells of MS patients expressed significantly less perforin (p = 0.011) and GrmB (p = 0.022) than did those of control subjects (Fig. 4A). Such a difference was not seen for GrmA or GrmK. Interestingly, after the addition of rhIL-2, there was a significant increase in perforin (65–84%, p = 0.027) and a strong trend for an increase in GrmB (87–96%, p = 0.052) in MS patients. Thus, the difference in perforin or GrmB content in HLA-B7/EBVRPP–specific CD8+ T cells between both categories vanished (Fig. 4A).

In contrast to HLA-B7/EBVRPP–specific CD8+ T cells, we found no difference with regard to cytotoxic granules content of HLA-A2/EBVGLC–, HLA-B8/EBVRak–, and HLA-B7/CMVTTPR–specific CD8+ T cells between the two categories of subjects, either with or without exogenous rhIL-2 (Fig. 4).

In MS patients, HLA-B7/EBVRPP–specific CD8+ T cell cytotoxic activity is rescued by the addition of exogenous IL-2

In the next step, we attempted to determine whether the decrease in perforin and GrmB in HLA-B7/EBVRPP–specific CD8+ T cells of MS patients affected their functional cytotoxicity. In a CFSE-based CTL functional assay, HLA-B7/EBVRPP–specific CD8+ T cells of MS patients showed, in the absence of rhIL-2, a decreased cytotoxic activity compared with cultures with rhIL-2 (p = 0.034; Fig. 5A). Moreover, the strength of this HLA-B7/EBVRPP–specific lysis was inversely proportional to the length of MS duration: the longer the disease, the lower the EBV-specific lysis (Fig. 5B, left panel). Adding exogenous rhIL-2 rescued the cytotoxic capacity of the EBVRPP-specific CD8+ T cells in MS patients (Fig. 5B, right panel).

In contrast, there was no such rescue effect of rhIL-2 on the cytotoxic activity in the control subjects (Fig. 5C). Contrasting again with EBV, there was no difference in the cytotoxic activity of HLA-B7/CMVTTPR–specific CD8+ T cells in the presence or absence of exogenous rhIL-2, and there was no correlation with disease duration (Fig. 5D, 5E). Similarly, there was no difference for the control subjects (Fig. 5F).

Proliferation of HLA-B7/EBVRPP–specific CD8+ T cells is not deficient in MS patients

Compared with controls, HLA-B7/EBVRPP–specific CD8+ T cells from MS patients proliferated at the same rate without rhIL-2, and they proliferated more when rhIL-2 was added (p = 0.04; Supplemental Fig. 4A). We can infer from these data that the relative lack of perforin and GrmB production by HLA-B7/EBVRPP–specific CD8+ T cells is not attributable to a deficit in the proliferation of EBV-specific tetramer+ cells. However, these data emphasize the high sensitivity of HLA-B7/EBVRPP–specific CD8+ T cells to rhIL-2. Indeed, upon addition of rhIL-2, the HLA-B7/EBVRPP–specific CD8+ T cells from MS patients proliferated more than did the HLA-A2/EBVGLC– and HLA-B8/EBVRak–specific CD8+ T cells (p = 0.004 and p = 0.02, respectively). No

FIGURE 2. Prevalence and magnitude of virus-specific HLA-A*0201, HLA-B*0702, or HLA-B*0801 tetramer+ CD3+CD8+ T cells among 220 study subjects carrying the appropriate HLA allele. (A) EBV-specific CD3+CD8+ T cells. (B) CMV-specific CD3+CD8+ T cells. Horizontal lines represent the median. *p < 0.05. CTRL, Control subjects; MS, MS patients; TET, tetramer.
such difference was seen for control subjects in either culture conditions. Finally, proliferation of HLA-B7/CMVTPR–specific CD8+ T cells was comparable between MS patients and control subjects under both culture conditions, although we acknowledge that the number of control subjects was low (Supplemental Fig. 4B).

**EBV DNA is not associated with deficient EBV-specific CD8+ T cell responses**

To determine whether the deficit in HLA-B7/EBV RPP–specific CD8+ T cells in MS patients was associated with a lack of viral control, we used real-time PCR to assess the presence of EBV DNA in the plasma and PBMC of the 19 MS patients and 11 control subjects who had been enrolled in the intracellular cytokine staining and CFSE functional CTL assays. EBV DNA was not detected in the plasma or in the PBMC of these 30 study subjects (data not shown).

**Discussion**

In this study, we first confirmed that MS patients express the HLA-B7 allele at a higher frequency than do control subjects (3, 36). This characteristic was shown to be due to linkage disequilibrium with the HLA-DR2 (DRB1*1501-DQB1*0602) allele, which increases the susceptibility to MS (3). However, our data suggest that HLA-B7 may be instrumental in the pathogenesis of MS. Indeed, using ex vivo tetramer staining, we show that, among HLA-B7+ study subjects, there is a significantly higher proportion of MS patients who exhibit an HLA-B7/EBV RPP–specific CD8+ T cell response compared with control subjects. Such a differential CD8+ T cell response is not present for the EBV peptides restricted by HLA-A2 or HLA-B8, nor is it present for HLA-A2– or HLA-B7–restricted CMV peptide epitopes. However, the magnitude of the CD8+ T cell response in MS patients is significantly lower for the HLA-B7/CMVTPR epitope and tends to be lower for the HLA-B7/EBV RPP epitope. With regard to the CD8+ T cell responses against the latter, other investigators found either no difference between MS patients and control subjects (37) or an increased magnitude of HLA-B7/EBV RPP–specific CD8+ T cells in MS patients (8), as reflected by the secretion of IFN-γ in an ELISPOT assay. However, a direct comparison is difficult, because the data on the magnitude of tetramer+ cells were obtained by gating on CD3+ and CD8+ T cells, whereas the ELISPOT assays were performed on total unfractionated PBMC.

Nevertheless, the discrepancy seen between the prevalence and the magnitude (Fig. 2) raises the question of a dysregulation of...
HLA-B7–restricted viral Ag presentation to CD8+ T cells in MS patients. Assessing in detail the ex vivo phenotype of viral-specific CD8+ T cells of MS patients and control subjects, we find a relative deficit in the secretion of IFN-γ and primarily IL-2 in HLA-B7–restricted EBV-specific CD8+ T cells but not in the four other HLA-restricted viral peptide epitope-specific CD8+ T cells. Upon Ag stimulation in 1-wk cultures, without addition of exogenous rhIL-2, HLA-B7/EBVRPP–specific CD8+ T cells of MS patients exhibit a defect in their ability to upregulate perforin and GrmB, which suggests that their memory HLA-B7/EBVRPP–specific CD8+ T cells are less efficient. In agreement with previous work showing a decreased secretion of IFN-γ by EBV-specific CD8+ T cells with an increase in MS duration (9), we show in this study that the HLA-B7/EBVRPP–specific CD8+ T cell–mediated cytotoxicity (but not HLA-B7/CMVTPR) also decreases with disease duration.

It was proposed that CD8+ T cells of MS patients may present an intrinsic deficit that would lead to suboptimal control of EBV replication (12, 14). Supporting this theory, polymorphism of the perforin gene is increased in MS patients, a feature that could account for a dysregulation of the cytotoxic activity of CD8+ T cells (38). Furthermore, an impaired function of CD8+ T cells leading to a decreased response against EBV, but not other viruses, does exist: X-linked lymphoproliferative disease. This condition is due to a dysfunction of the signaling lymphocytic activation molecule-associated protein involved specifically in the response to EBV (39). Affected patients exhibit a very high susceptibility to EBV infection, often with a fatal outcome. Thus, the question arises whether MS patients could be affected by a sort of attenuated X-linked lymphoproliferative disease. Our data do not suggest that there is a global or massive EBV-specific CD8+ T cell deficiency in MS patients. Indeed, we find no difference between MS patients and control subjects in terms of an HLA-A2– or HLA-B8–restricted EBV-specific CD8+ T cell response. Furthermore, we do not detect EBV DNA either in the plasma or PBMC of MS patients or control subjects. This result, which contrasts with that of other investigators (10, 13), could be due to methodological differences. Furthermore, because we tested one time point, which was not always the same as the cellular assay time point, we may have missed a transient increase in EBV viral load. Nevertheless, our data suggest that the
impaired HLA-B7–restricted EBV-specific CD8+ T cell responses are not associated with an obvious increase in EBV viral load. Therefore, based on our findings, it is the interaction between CD8+ T cells and the presentation of the EBVpp epitope by the HLA-B7 allele that is suboptimal in MS patients. It was recently shown that the HLA class I molecule is expressed at the same level in MS patients and controls (40), suggesting that the dysregulation of the HLA-B7/EBV-specific CD8+ T cell response is not due to a quantitative deficit of this molecule on the surface of APCs. In fact, our data suggest that a defective costimulation may explain this phenomenon. Indeed, the content in perforin and GrmB, as well as the cytotoxic function of HLA-B7/EBVpp–specific CD8+ T cells, is fully restored if rhIL-2 is added to the 1-wk culture, suggesting that the endogenous production of IL-2 in MS patients is not sufficient to fully restore the proliferative capacity of CD8+ T cells (42). Thus, the question is whether there is a deficiency in IL-2 in MS.

Specifically, ex vivo analysis of cytokine secretion by HLA-B7–restricted EBVpp-stimulated CD8+ T cells shows that, although ~50% of the CD8+ T cells secrete IFN-γ and IL-2 in both MS patients and control subjects, the amount of IL-2 secreted by a given HLA-B7–restricted EBVpp-specific CD8+ T cell is lower in MS patients. We posit that a deficit in the synthesis of endogenous IL-2 by CD8+ T cells explains the lower synthesis of perforin and GrmB and the lower cytotoxicity by HLA-B7/EBVpp–specific CD8+ T cells during the rhIL-2–deprived 1-wk stimulation. Contrasting to some degree with these data, it was shown that MS patients have increased IL-2 concentrations in serum and cerebrospinal fluid (43), whereas at the cellular level, production of IL-2 varies with disease activity (44) and Ag-specific or -aspecific stimulation (16, 45).

Interestingly, genome-wide association studies identified polymorphisms of IL2RA associated with several autoimmune diseases, including MS (reviewed in Ref. 46). Indeed, two single-nucleotide polymorphisms have been identified within the IL2RA of MS patients (47–49). Further, functional studies demonstrated that the diverse polymorphisms of the IL2RA gene contributed independently to MS susceptibility (48). Because we did not look at the IL2RA molecule in this study, we cannot rule out that this mechanism is involved in the dysregulation of HLA-B7/EBVpp–specific CD8+ T cells. Additional studies are warranted to examine this particular aspect.

One can wonder whether the fact that the HLA-B7–restricted EBVpp is an epitope of a latent protein (EBNA3A) and the EBV peptides restricted by HLA-A2 and HLA-B8 are epitopes of lytic proteins (BMLF1 and BZLF1) may account for the observed differences. Indeed, we cannot rule out that the CD8+ T cell response against latent peptide epitopes presented by other alleles would have shown differences between MS patients and control subjects. However, we do not think that our data can be solely explained by this phenomenon. Indeed, the EBVpp peptide used in this study is one of the most immunogenic of the EBV HLA-B7–restricted peptides (50, 51). Second, in a recent work, we showed that the cytotoxic activity of CD8+ T cells against latent peptides in general (also restricted by other HLA class I) was greater than against lytic epitopes (11). Finally, the lower magnitude of the HLA-B7/CMVpp–specific CD8+ T cell response, ex vivo, in MS patients further points toward a deficient presentation of viral peptide epitope by the HLA-B7 molecule.

In conclusion, our data suggest that, although there is a higher proportion of HLA-B7+ MS patients who harbor EBVpp–specific CD8+ T cells, this trimolecular interaction is suboptimal. The fact that IL-2 production is decreased in HLA-B7–restricted EBVpp–specific CD8+ T cells and that the magnitude of the CD8+ T cell

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**FIGURE 5.** Upon stimulation with HLA-B7/EBVpp peptide, MS patients exhibit a deficit in CD8+ cytotoxic activity. CFSE-based cytotoxic CTL assay of HLA-B7/EBVpp–specific CD8+ T cells in the absence or presence of rhIL-2 in MS patients (A) or control subjects (C). (B) In the absence of rhIL-2, HLA-B7/EBVpp–specific CD8+ T cell cytotoxicity is inversely correlated with MS duration (left panel); however, such a correlation is lost when cells are cultured in the presence of rhIL-2 (right panel). Similar experiments were performed for HLA-B7/CMVpp–specific CD8+ T cells in MS patients (D) and control subjects (F). (E) Absence of correlation of HLA-B7/CMVpp–specific CD8+ T cell cytotoxicity with MS disease duration. Each symbol represents one subject; horizontal lines represent the median. *p < 0.05. CTRL, Control subjects; MS, MS patients; yr, years.
response against another HLA-B7–restricted viral peptide epitope (CMV\textsubscript{pp65}) is also diminished in MS patients points toward a deficient presentation of viral epitopes by this specific class I HLA. This observation is particularly intriguing considering the fact that HLA-B7 is more prevalent in MS patients, and EBV is associated with MS.

**Acknowledgments**

We thank K. Ellefsen-Lavoie and E. Medjitna for providing the HLA-B7/EBVpp and HLA-B7/CMV\textsubscript{pp65} tetramers and G. Le Golf and C. Campanile for invaluable help in enrolling patients and obtaining blood samples. We also are grateful to P. Meylan and S. Chappuis, who performed quantification of virus-specific Abs. We thank the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA) for providing the HLA-B8/EBV\textsubscript{RAK} tetramer.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure 1

Ex vivo content of perforin, GrmB, GrmA and GrmK in EBV- and CMV-specific CD8+ T cells.

A) In this representative MS patient, the gating strategy is illustrated by the content in perforin, GrmA, GrmB and GrmK in HLA-B7/CMV_{TPR} tetramer+ cells.

B) Percentage of perforin, GrmB, GrmA and GrmK in HLA-A2/EBV_{GLC}, HLA-B7/EBV_{RPP} and HLA-B8/EBV_{RAK} and C) HLA-A2/CMV_{NLV} and HLA-B7/CMV_{TPR} tetramer+ cells. Each dot represents one study subject and horizontal bars represent the median. MS, MS patients; CTRL, control study subjects.
**Supplemental Figure 2**

A) EBV- and B) CMV-specific tetramers. On the x-axis, the 16 possible combinations of the different markers are displayed. On the y-axis, the percentages of the perforin/granzyme cell subsets within virus-specific CD8+ T cells are indicated. Each dot represents one subject. MS patients are depicted in blue and control study subjects in red. The pie charts summarize the data, each slice corresponding to the proportion of a given combination of cytolytic granules in virus-specific CD8 T cells. MS, MS patients; CTRL, control study subjects.

_Detailed analysis of the populations of tetramer+ cells in terms of content in cytolytic granules for each individual tetramer._
The pattern of differentiation of tetramer-positive CD8+ T cells is similar in MS patients and control subjects for both viruses. Percentage of A) CCR7-CD8+ T cells, B) CD57+CD8- T cells specific for HLA-A2/EBVGLC, HLA-B7/EBV RPP and HLA-B8/EBV RAK, HLA-A2/CMVNLV and HLA-B7/CMV TPR. C) MFI of PD-1 CD8+ HLA-B7/EBV RPP-specific CD8+ T cells. Each dot represents one subject and horizontal bars represent the median. MS, MS patients; CTRL, control study subjects; MFI, mean fluorescence intensity; *p<0.05.
Supplemental Figure 4

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Tetramer+ CD8+ T cells proliferated more in MS patients as compared to control subjects upon antigen stimulation. A) HLA-A2/EBV<sub>GLC</sub>, HLA-B7/EBV<sub>RPP</sub> and HLA-B8/EBV<sub>RAK</sub> and B) HLA-B7/CMV<sub>TPR</sub> tetramer fold in MS patients and control subjects after short-term stimulation (7 d). Tetramer fold increase was calculated by comparing tetramer frequency before and after stimulation in CD3+CD8+ T cells for each study subject. MS, MS patients; CTRL, control study subjects; *p<0.05.