Journal of Neuroimmunology 225 (2010) 167–170

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short communication

Cytokine mRNA profile of Epstein–Barr virus-stimulated highly differentiated T cells in multiple sclerosis: A pilot study

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ARTICLE INFO

Article history:
Received 10 December 2009
Received in revised form 26 March 2010
Accepted 20 April 2010

Keywords:
Multiple sclerosis
Epstein–Barr virus
CD8+ T cells
Cytokines

ABSTRACT

The reason why EBV-specific cellular immune responses are abnormal in multiple sclerosis (MS) patients is still missing. In this exploratory pilot study, we assessed IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, TGF-β1 and FOXP3 mRNA expression in EBV-stimulated highly differentiated T cells (T_{HD}) of MS patients and healthy controls (HC). We found increased levels of IFN-γ and IL-4 mRNA in CD8+ T_{HD} cells of MS patients. All the other tested molecules were expressed similarly in MS patients and HC. Interestingly, increased IFN-γ and IL-4 suggest that the control of EBV replication may be insufficient in MS patients.

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1. Introduction

Increased humoral and cellular immune responses against the γ-herpesvirus Epstein–Barr (EBV) have been consistently reported in multiple sclerosis (MS) patients (Ascherio and Munger, 2007). The cause of such high immune responses remains unknown. We have recently reported a strong EBV-, but not cytomegalovirus (CMV)-specific, IFN-γ mediated, CD8+ T cell response in the blood of patients with early MS as compared with healthy controls (HC) (Jilek et al., 2008). We have also found an intrathecal enrichment in EBV-specific CD8+ cytotoxic T lymphocytes in patients with early MS (Jaquiéry et al., 2010). Thereby, our data speak in favour of an increased immunity towards EBV in MS patients, at least in the early stage of the disease. However, differing from our observations, others have postulated that there was a defect in EBV-specific CD8+ T cell responses (Lindsey and Hatfield, 2008; Pender et al., 2009).

To explore whether there is indeed a skewed functional cellular immune response against EBV in MS patients, we examined the mRNA profile of both pro-inflammatory (IL-1β, IL-2, IL-6, IL-17 and IFN-γ) and anti-inflammatory (IL-4, IL-10, TGF-β1 and FOXP3) cytokines and transcription factors. These molecules are important mediators of immune responses and have all been suggested to be significant players in MS (McFarland and Martin, 2007). Thus, if there was a dysregulated immune response against EBV in MS patients, it is likely that it would be mediated by one or more of these cytokines/transcription factors. Such as in previous studies, CMV was used as a control virus. Indeed, like EBV, it is a herpesvirus that can establish latent infections. Moreover, CMV is neurotropic, but has not been associated with MS (Ascherio and Munger, 2007). In order to focus on the most relevant CD4+ and CD8+ T cells, we sorted them according to the expression of CCR7, a chemokine receptor controlling homing to secondary lymphoid organs. Indeed, CCR7+ T cells are composed of naïve and central memory cells and are poorly differentiated and therefore lack the capacity for immediate responses to antigens. By contrast, highly differentiated T cells (T_{HD}) do not express CCR7, but express receptors for migration to inflamed tissues and display immediate effector functions as revealed by their capacity to produce IFN-γ and perforin (Sallusto et al., 1999). Secretion of cytokines upon antigen stimulation is therefore concentrated in T_{HD} cells (Champagne et al., 2001). Finally, it is thought that CD8+ T_{HD} cells play an important role in the pathogenesis of MS since they are enriched in the CSF of patients with recent onset of MS (Jilek et al., 2007).

2. Materials and methods

2.1. Study subjects

We enrolled ten MS patients (median disease duration: 3.6± 3.2 years), including three with a clinically isolated syndrome and seven with relapsing–remitting MS (Polman et al., 2005), as well as 11 age- and sex-matched HC. These 21 subjects had previously been identified as harbouring detectable EBV-specific CD4+ and CD8+ T cell

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doi:10.1016/j.jneuroim.2010.04.011

responses (Jilek et al., 2008). Of this cohort, seven MS patients and four HC also exhibited vigorous CMV-specific CD4+ and CD8+ T cell responses. Indeed, we were not able to find all subjects responding to both EBV and CMV, which reflects the lower infection rate of CMV (50%) as compared with EBV (95%) in the general population (Ascherio and Munger, 2007). All subjects gave written consent according to our institution's review board guidelines. None had received immunomodulatory or immunosuppressive treatment within three months prior to enrolment.

2.2. Peripheral blood mononuclear cell (PBMC) stimulation

For each condition, 10^7 fresh PBMC were stimulated with purified EBV lysate (ABI Inc.), purified CMV lysate (ABI Inc.), pool of immunodominant eight- to fifteen-mer peptide EBV epitopes (JPT Peptide Technologies) or pool of immunodominant eight- to fifteen-mer peptide CMV epitopes (JPT Peptide Technologies), each at 1 μg/ml, in RPMI (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) for 17 h at 37 °C. The EBV and CMV immunodominant peptides used for stimulation have already been described (Jilek et al., 2008). We have previously reported that viral lysates elicit specifically CD4+ T cells, whereas pools of peptide viral epitopes elicit CD8+ T cell responses (Jilek et al., 2008).

2.3. Sorting

Stimulated PBMC were incubated with anti-CD3-PE, anti-CD4-APC (both from Becton Dickinson), anti-CD8-PB (Dakocytomation) and anti-CCR7-FITC (R&D) for 20 min at 4 °C, washed in PBS and resuspended at four to six millions/ml in PBS. For each condition of stimulation and in each category of study subjects, 10^5 cells of each subset (CD3+ CD4+ CD8− CCR7+, CD3+ CD4+ CD8− CCR7−, CD3+ CD8+ CD4− CCR7+ and CD3+ CD8+ CD4− CCR7−) were sorted at 4 °C using a BD FACSAria Flow Cytometer (Becton Dickinson). The grade of purity ranged between 95 and 98%.

2.4. Quantitative real-time PCR (qRT-PCR)

RNA from 10^5 sorted cells was extracted (RNeasy Mini Kit, Qiagen) and reverse transcribed into cDNA (QuantiTect Reverse Transcription Kit, Qiagen). To avoid variations due to different reverse transcription efficiencies, three reactions were set up and pooled for each sample before qRT-PCR analysis (ABI Prism 7700 SDS, Applied Biosystems). The QuantiFast SYBR Green PCR Kit (Qiagen) was used as the amplification mix in combination with QuantiTect Primer Assays (Qiagen). To avoid contamination of reagents and samples, a strict work-flow was followed and distinct pre- and post-PCR areas were used under stringent regulations. 18S ribosomal RNA (rRNA) was used as an endogenous control, since it does not fluctuate upon lymphocyte stimulation (Roge et al., 2007). The expression of each cytokine mRNA was calculated according to the following formula: Arbitrary Units (A.U.) $= 100 \times [1/(Ct_{cytokine} - Ct_{18S\ rRNA}) - 4.543]$ (Jilek et al., 2004), where Ct represented the cycle number at which the SYBR green fluorescence crossed the threshold value and where 4.543 represents the default value set for undetectable samples. A melting curve analysis was performed to ensure specificity of the PCR products. PCR efficiencies ranged between 90 and 100% and were similar for all target genes.

2.5. Enzyme-linked immunospot (ELISPOT) assay

The frequency of EBV-specific CD8+ T cells secreting IFN-γ was assessed by using an ELISPOT assay, such as previously described (Jilek et al., 2008). Briefly, 200,000 PBMC in 200 μl of RPMI supplemented with 10% fetal calf serum were incubated in triplicates in the presence of EBV immunodominant peptides at 1 μg/ml. Peptide-free medium and PHA-L (5 μg/ml) served as negative and positive controls, respectively. Responses were expressed as net spot-forming cells per 10^6 PBMC. The assay was considered experimentally valid if the spot-forming cells, in the absence of peptide, was lower than 40 per 10^6 cells. These background values were subtracted from the peptide-stimulated data before analysis. Of note, sufficient cell numbers to perform the ELISPOT assay were available from 9 MS patients and 7 HC.

2.6. Statistical analysis

Differences among two or more groups were tested using Mann–Whitney, respectively Kruskal–Wallis test. Correlations were analysed using the Spearman's test. A $P<0.05$ was considered significant.

3. Results

3.1. IFN-γ and IL-4 mRNA are differentially expressed in EBV-stimulated CD8+ T_{HD} cells of MS patients

Virus-stimulated PBMC were sorted according to the strategy presented in Fig. 1A for one representative patient. We found that IFN-γ mRNA levels were significantly raised in EBV- ($P=0.02$; Fig. 1B), but not CMV-stimulated ($P>0.4$; Fig. 1C) CD8+ T_{HD} cells of MS patients, as compared with HC. IFN-γ secretion by EBV-specific CD8+ T cells, such as determined by the ELISPOT assay in PBMC, correlated significantly with the EBV-specific IFN-γ mRNA expression found in EBV-stimulated CD8+ T_{HD} cells, as assessed by qRT-PCR ($r_s=0.75$, $P=0.0008$; Fig. 1D). EBV-stimulated CD8+ T_{HD} cells from MS patients also expressed significantly increased levels of IL-4 mRNA as compared with HC ($P=0.04$; Fig. 1B).

Regarding the mRNA coding for IL-2, IL-10, FOXP3 and TGF-β1, no difference in expression was found between both categories of study subjects for EBV- or CMV-stimulated CD8+ T_{HD} cells. IL-1β, IL-6 and IL-17 mRNA were not detected in EBV-stimulated CD8+ T_{HD} cells of both MS patients and HC. Similarly, CMV-stimulated CD8+ T_{HD} cells did not express IL-6 nor IL-17 mRNA in both categories of study subjects, whereas IL-1β was detected in 1/7 MS patient and 2/4 HC (Fig. 1B and C and data not shown). Virus-stimulated CCR7+CD8+ T cells expressed lower cytokine mRNA levels than CD8+ T_{HD} for all cytokines studied, confirming that T_{HD} cells were the appropriate subpopulation of cells for our study (data not shown). Finally, we did not find any differences in terms of cytokine secretion by EBV- or CMV-stimulated CD4+ T_{HD} cells between MS patients and HC (data not shown).

4. Discussion

In this exploratory study, we found an increase of mRNA coding for IFN-γ and IL-4 in EBV-stimulated CD8+ T_{HD} cells of MS patients as compared with HC. However, differences were seen neither in CMV-stimulated CD8+ T_{HD} cells nor in EBV- or CMV-stimulated CD4+ T_{HD} cells. These data confirm previous results (Jilek et al., 2008), and show that IL-4 also seems to be enhanced in the EBV-stimulated CD8+ T_{HD} cells of MS patients. This is of particular interest since both over-expression of IL-4 (Zekry et al., 2002) and monofunctional IFN-γ T cell responses (Harari et al., 2006) are associated with lack of control of virus replication and high viral load levels. Therefore, we may hypothesize that these dysregulated EBV-specific CD8+ T cells might drive autoimmune responses by cross-reacting with myelin antigens, the so called molecular mimicry mechanism, or by bystander activation of autoreactive cells (Munz et al., 2009). Nevertheless, to date, it is not possible to affirm whether the high EBV-specific immune responses are beneficial or detrimental to MS patients. An animal model is needed to address this question. However, such a model is difficult to establish as EBV does not infect mice, which calls for a humanized animal model (Munz and Becher, 2008; Yajima et al., 2008).

In contrast with EBV-, we did not find differences in the cytokine profile of CMV-stimulated CD8+ T_{HD} cells between MS and HC. However, pertaining to the low number of HC responding to CMV-

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