

Characterization of highly differentiated viral-specific T cells in multiple sclerosis using quantitative real-time PCR

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INTRODUCTION:

Several epidemiological studies have shown an association between Epstein-Barr virus (EBV), a γ -herpesvirus, and multiple sclerosis (MS) [1]. In contrast to the well studied EBV-specific humoral immune response in MS patients, the EBV-specific cellular immune response was less well characterized. Our group has recently found that there is a higher frequency of IFN- γ secreting EBV-specific CD8+ T cells in early MS patients (see poster of S. Jilek). These results are in line with results from Lünemann and Höllsberg, who respectively showed that EBNA1-specific memory CD4+ T cells are increased in MS patients [2] and that EBV-specific CD8+ T cells are found at an increased frequency in MS patients when compared to healthy controls [3]. Cytomegalovirus (CMV), another herpesvirus which has not been associated with MS, was used as a control in our study.

We have previously shown that highly differentiated (CCR7-) CD8+ T cells were enriched in the cerebrospinal fluid of early multiple sclerosis (MS) patients, thus arguing for a role of these cells at the onset of MS [4]. Therefore, in this study, we examined by quantitative real-time PCR (qRT-PCR) the cytokine mRNA expression under basal conditions in bulk CD4+ and CD8+ T cells and after EBV or CMV stimulation in highly differentiated CD4+ and CD8+ T cells. We used this method rather than intracellular cytokine staining since qRT-PCR is able to detect very low levels of these proteins in unstimulated lymphocytes.

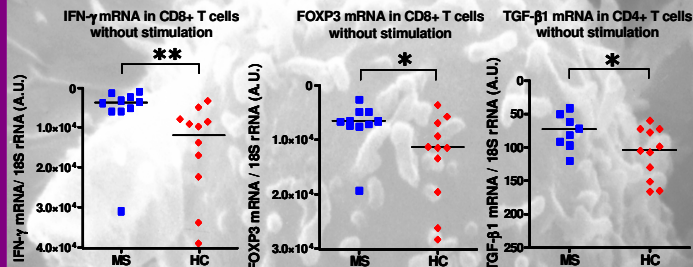
MATERIAL AND METHODS:

Patients: We enrolled 10 inflammatory MS patients (3 patients with clinically isolated syndrome and 7 with relapsing-remitting MS) and 11 age-matched healthy controls (HC) with a strong EBV-specific T cell response. Of them, 8 MS patients and 6 HC were also identified to harbour a strong CMV-specific T cell response. All study subjects gave their informed consent according to the IRB of our hospital. After blood draw, PBMC were immediately processed.

Methods: We stimulated PBMC with either viral lysate or immunodominant peptide epitopes to elicit a CD4+ or a CD8+ response, respectively. After 17h of stimulation at 37°C, these T cells were sorted according to their CCR7 staining, RNA extracted from each cell subset, and cDNA synthesized. Expression of pro-inflammatory (IFN- γ , IL-2, IL-1 β) and of anti-inflammatory (FOXP3, IL-4, TGF- β 1) cytokines was assessed by qRT-PCR. 18S rRNA was used as an endogenous control to normalize for differences in the amount of cDNA between samples. Relative quantification of the transcripts was performed using the comparative $2^{-\Delta\Delta Ct}$ method as described by Livak [5]. The difference of expression between target genes and the reference gene (18S rRNA) corresponded to the basal level of gene expression in medium-stimulated cells or to the total level of gene expression in virus-stimulated cells.

Statistics: Data were LOG-transformed before statistical analysis to render them normally distributed. Two-tailed unpaired t test was used.

MS effect on basal cytokine mRNA expression

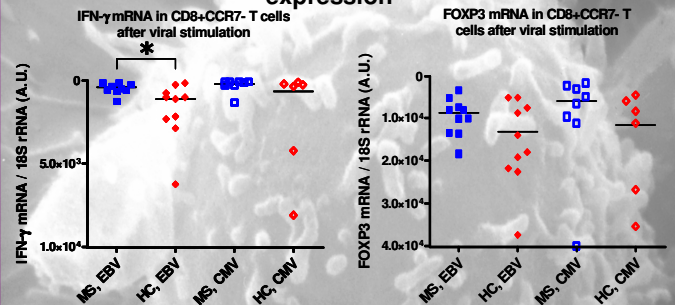


IFN- γ and FOXP3 (in CD8+ T cells) and TGF- β 1 (in CD4+ T cells) mRNA expressions are increased in unstimulated cells of MS patients compared with HC.

Cytokine mRNA expression was assessed by quantitative real-time PCR in medium-stimulated CD8+ (MS: n=10, HC: n=11) and CD4+ (MS: n=8, HC: n=11) T cells. Horizontal bars represent geometric mean. *p<0.05, **p<0.01 (unpaired t test).

Over the six cytokines for which mRNA expression was assessed, we found an increased expression of the pro-inflammatory cytokine IFN- γ in CD8+ T cells of MS patients. Unexpectedly, the anti-inflammatory cytokines FOXP3 in CD8+ T cells and TGF- β 1 in CD4+ T cells were also found to be increased in MS patients.

Viral effect on total cytokine mRNA expression



IFN- γ mRNA total expression is increased in highly differentiated CD8+ T cells of MS patients after EBV but not CMV stimulation compared with HC. FOXP3 levels are similar in MS and HC after EBV and CMV stimulations in these cells.

Cytokine mRNA expression was assessed by quantitative real-time PCR in EBV (MS: n=10, HC: n=10) or CMV (MS: n=8, HC: n=6) immunodominant peptide epitopes stimulated CD8+CCR7- T cells. Horizontal bars represent geometric mean. *p<0.05 (unpaired t test).

Confirming results obtained by ELISPOT assays (see poster of S. Jilek), we found that EBV-stimulated highly differentiated CD8+ T cells harboured more IFN- γ mRNA in MS patients than in HC, whereas such differences were not found in CMV-specific CD8+ T cells. However, FOXP3 mRNA expression in CD8+CCR7- T cells (and TGF- β 1 in CD4+CCR7-) did not significantly differ between MS and HC after either EBV or CMV stimulation.

CONCLUSION:

It is now widely accepted that EBV is linked to MS [1]. EBV-specific humoral and cellular immune responses were shown to be increased in MS patients [1, 2, 3, poster of S. Jilek]. On the other hand, dysregulation of cytokine secretion was recently demonstrated in MS patients [6, 7, 8]. However, the majority of these studies have been performed in the serum or PBMC of patients, thus limiting the identification of the cells involved.

Here, we show that basal IFN- γ mRNA expression is significantly more elevated in the CD8+ T cells of MS patients compared to HC, suggesting that CD8+ T cells are already more activated in MS patients than in HC before any stimulation.

Similarly, but more surprisingly, the anti-inflammatory cytokines TGF- β 1 and FOXP3 were increased in CD4+ and CD8+ T cells respectively. This elevated basal anti-inflammatory cytokine expression could be explained by the fact that all the patients except one were in remission or that there is a feedback mechanism trying to reduce the elevated IFN- γ basal expression.

Furthermore, we confirmed our previous results [poster of S. Jilek]. Indeed, we found a significantly higher IFN- γ mRNA expression after EBV stimulation in CD8+CCR7- T cells, but not in EBV-stimulated CD4+CCR7- or CMV-stimulated CD4+CCR7- and CD8+CCR7- cells. However, there was no difference in the expression of the other four cytokines (IL-2, IL-1 β , FOXP3, TGF- β 1) after EBV or CMV stimulation in our study subjects. This reinforces the link between EBV, IFN- γ secreting effector T cells and MS.

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