

# Increased Intrathecal Immune Responses to Epstein-Barr Virus in Early Multiple Sclerosis\*

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## INTRODUCTION:

Epstein-Barr virus (EBV) has been consistently associated with multiple sclerosis (MS) by examining cellular and humoral immune responses in the blood [1, 2]. If EBV indeed plays a role in the pathogenesis of MS, it is likely that its signature would be maximal in the central nervous system. Yet, relatively few authors have examined the relationship between EBV and MS in this compartment. Although these data are controversial [3], EBV-infected B cells located in neo-follicles in the meninges have been detected in patients with MS, but not with other inflammatory neurological diseases. Activated CD8+ T cells present in the vicinity of these neo-follicles were suggestive of ongoing cytotoxic activity against EBV-infected B cells [4]. In addition to raising the question of the involvement of EBV in MS, these data point toward a pathogenic role of CD8+ T cells in this disease. Linking CD8+ T cells and EBV, we have shown that early MS patients harbor an increased EBV-specific, IFN- $\gamma$  mediated, CD8+ T cell response in the blood [2]. We have also reported that there was an enrichment in highly differentiated CD8+ T cells in the cerebrospinal fluid (CSF), however we did not address their specificity [5]. Altogether, these data suggest that an abnormal EBV-specific immune response may take place in the central nervous system. Thus, here, we examined the EBV-specific humoral and cellular immune responses in the CSF and blood of patients with early MS or other neurological diseases, separated into inflammatory (OIND) and non-inflammatory (NIND) groups. The neurotropic herpesvirus cytomegalovirus (CMV), a virus which is not considered to be associated with MS, served as a control.

## MATERIAL AND METHODS:

**Patients:** We obtained paired blood and CSF samples of 123 patients, including patients who had their first symptoms of MS less than one year prior to our assays (early MS) and patients with OIND and NIND.

**Serologies:** anti-EBV IgG were measured with a multiplexed immunoassay (Luminex) and anti-CMV IgG with an ELISA in the 123 study patients.

**Effector cells:** In patients of the cellular immune response arm, PBMC and CSF cells were stimulated with EBV- or CMV-specific pools of immunodominant peptide epitopes known to elicit CD8+ T cells, and cultured for 11-14 days in the presence of exogenous IL-2.

**Functional CFSE cytotoxic T lymphocyte (CTL) assay:** Target cells were prepared by staining autologous PBMC with CFSE and loading them with EBV or CMV peptides. After 18h of incubation with increasing ratios of effector cells, surviving target cells were quantified by flow cytometry.

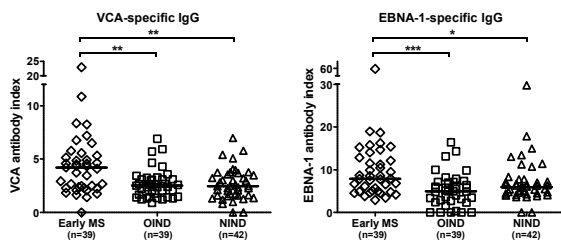
**Tetramer staining:** Effector cells from the blood and CSF of a subset of patients were stained with the HLA-A\*0201/BMLF-1<sub>GLCTLVAMIL</sub> or the B\*0702/EBNA-3A<sub>RPPFIRRL</sub> tetramer. BMLF-1 is a protein which is part of the lytic cycle of EBV, while EBNA-3A is a latent protein of EBV.

## Clinical and laboratory data of the cohort

	Early MS (n=40)	OIND (n=39)	NIND (n=44)
Age in years <sup>1</sup>	33 ± 11	44 ± 20	44 ± 20
Disease duration in months <sup>1</sup>	1.2 ± 3.0	1.2 ± 2.0	1.3 ± 12.3
CSF leucos per $\mu$ l <sup>1</sup>	4 ± 5	5 ± 6	1 ± 0.4
Intrathecal synthesis of IgG	36/40	15/39	1/44
Patients in cellular immune response arm	17/40	20/39	18/44

<sup>1</sup>Numbers represent median  $\pm$  interquartile range.

## Increased Intrathecal VCA- and EBNA-1-specific IgG in Patients with Early MS

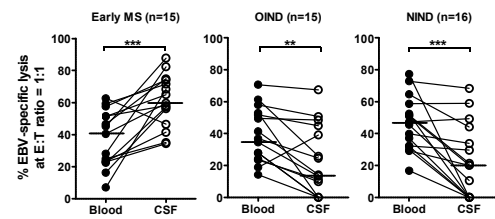


### EBV-specific humoral immune responses in the CSF of study patients.

Plasmas and CSF were assayed for anti-VCA IgG and anti-EBNA-1. The respective antibody indexes (AI), i.e. the recruitment of a given virus-specific antibody in the CSF as compared to the blood, were calculated with the Reiber's formula [6]. Horizontal bars represent the median values. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001 (Kruskal-Wallis and Mann-Whitney ranked tests).

Patients with early MS had increased VCA and EBNA-1 AI as compared to those with OIND and NIND.

## High Intrathecal EBV-specific CD8+ Cytotoxic T lymphocyte Activity in Patients with Early MS

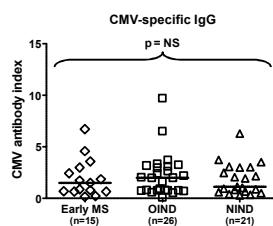


### EBV-specific CD8+ CTL activities in the blood and CSF of study patients.

In peptide pool responsive patients (15/17 early MS, 15/20 OIND and 16/18 NIND), determination of EBV-specific CD8+ CTL activity in the blood and CSF was done using effector cells in a functional CFSE CTL assay. Horizontal bars represent the median values. E:T ratio, effector:target ratio. \*\*p<0.01, \*\*\*p<0.001 (Wilcoxon ranked test).

There was a significant intrathecal enrichment in EBV-specific CD8+ CTL in patients with early MS. By contrast, this response was significantly lower in the CSF than in the blood of patients with OIND and NIND.

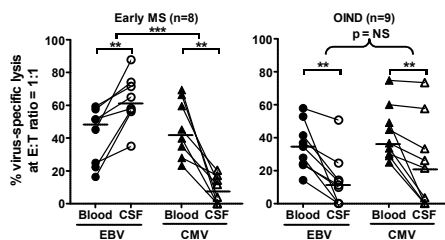
## Intrathecal CMV-specific Immune Responses Are Similar in All Categories of Patients



### CMV-specific humoral immune responses in the CSF of study patients.

Plasmas and CSF were assayed for anti-CMV IgG. Antibody indexes (AI), i.e. the recruitment of a given virus-specific antibody in the CSF as compared to the blood, were calculated with the Reiber's formula [6]. Horizontal bars represent the median values. NS, not significant (Kruskal-Wallis ranked test).

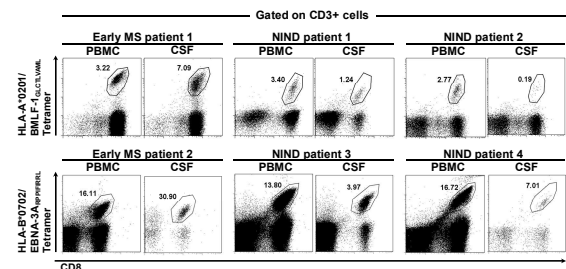
Contrary to EBV AI, patients with early MS had similar CMV AI as patients with OIND and NIND.



### CMV-specific cellular immune responses in the blood and CSF of study patients.

Of the 55 patients for whom the cellular immune responses were assessed, 8/13 early MS, 9/13 OIND and 0/1 NIND patients i) had enough cells in their CSF (>6000) to set up parallel EBV and CMV cultures and ii) were responsive to both EBV and CMV peptide pools. Therefore, in these 17 patients, the parallel comparison of EBV- and CMV-specific CD8+ CTL response was assessed using effector cells in a functional CFSE CTL assay. Horizontal bars represent the median values. E:T ratio, effector:target ratio. NS, not significant, \*\*p<0.01, \*\*\*p<0.001 (Wilcoxon ranked test and Fisher's exact test).

All eight early MS patients displayed an intrathecal enrichment in EBV-specific CD8+ CTL, but none of these eight exhibited any CSF recruitment in CMV-specific CD8+ CTL. By contrast, the response of the nine OIND patients in the CSF was similarly lower than in the blood for both EBV and CMV.



### EBV tetramer staining in blood and CSF effector cells of study patients.

CSF and blood effector cells were stained with the HLA-A\*0201/BMLF-1<sub>GLCTLVAMIL</sub> and B\*0702/EBNA-3A<sub>RPPFIRRL</sub> tetramers in three HLA-A\*0201+ study patients (1 early MS and 2 NIND) and three HLA-B\*0702+ study patients (1 early MS and 2 NIND), respectively.

PBMC and CSF effector cells generated upon stimulation with EBV peptide pool and cultured for 11 to 14 days contained tetramer-positive cells, confirming that our pool of EBV peptides indeed led to the clonal expansion of epitope-specific CD8+ CTL. Interestingly, these tetramer data are consistent with the functional CFSE CTL assay, since there was enrichment in tetramer-positive cells in the CSF of the two early MS, but in none of the four NIND patients.

## CONCLUSION:

- Intrathecal humoral immune responses to VCA and EBNA-1 were increased in patients with early MS.
- In parallel, EBV-specific CD8+ CTL were enriched in the CSF of patients with early MS.
- By contrast, CMV-specific humoral and cellular immune responses were similar in early MS, OIND and NIND patients. Moreover, there was no indication of an increased EBV-specific CD8+ CTL activity in the CSF of patients with other neurological diseases, be they inflammatory or not. These strict controls rule out the possibility that the high EBV-specific immune responses observed in the CSF of early MS patients was due to a mere aspecific inflammation-driven process.
- These data strengthen the link between EBV and MS, in particular in the early phase of the disease.

## REFERENCES:

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## ACKNOWLEDGEMENTS:

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