

**Low cytomegalovirus seroprevalence in early multiple sclerosis: a case  
for the “hygiene hypothesis”?**

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## ABSTRACT

**Background and purpose:** Cytomegalovirus (CMV) infection has been recently associated with a lower multiple sclerosis (MS) susceptibility, remaining controversial whether it has a protective role or is merely an epiphenomenon related to westernization and early-life viral infections. We aimed to evaluate whether CMV serostatus may differ in early MS as compared to non-early MS patients, analyzing the putative association of this virus with MS clinical course and humoral immune responses against other herpesviruses.

**Methods:** Multicentric analysis of 310 MS patients (early MS, disease duration  $\leq 5$  years, n=127) and controls (n=155), evaluating specific humoral responses to CMV, Epstein-Barr virus (EBV) and human herpesvirus-6 (HHV-6), as well as T- and Natural Killer (NK)-cell immunophenotypes.

**Results:** CMV seroprevalence in early MS was lower than in non-early MS or controls (p<0.01), being independently associated with disease duration (odds ratio 1.04, 95% confidence interval 1.01-1.08, p<0.05). CMV+ MS patients displayed increased proportions of differentiated T-cells (CD27-CD28-, CD57+, LILRB1+) and of NKG2C+ NK-cells, which were associated with a lower disability in early MS (p<0.05). CMV+ early MS patients had an age-related decline in serum anti-EBNA-1 antibodies (p<0.01), but no CMV-related differences in anti-HHV-6 humoral responses.

**Conclusions:** Low CMV seroprevalence was observed in early MS patients. Modification of MS risk attributed to CMV might be related to the induction of differentiated T- and NK-cell subsets and/or modulation of EBV-specific immune responses at early stages of the disease.

## INTRODUCTION

Multiple sclerosis (MS) is believed to be caused by a complex interplay between genetic and environmental risk factors (e.g. herpesvirus infections). Most evidences support a role of Epstein-Barr virus (EBV), a gamma-herpesvirus almost universally present in MS patients [1]. Other herpesviruses (e.g., human herpesvirus-6 (HHV-6)) have been also related to MS [2]. By contrast, recent studies have associated cytomegalovirus (CMV) infection with a decrease in MS susceptibility [3-5], although a causal relation and the mechanisms underlying a putative protective effect remain controversial [6]. Notwithstanding, the influence of herpesviruses in MS seems to be more marked at early stages of the disease, as evaluated by specific-immune responses [7-9].

CMV is a beta-herpesvirus that establishes a lifelong infection in 60-100% of human populations. Substantial immunological resources are devoted to control CMV infection, leading to an “inflation” of terminally differentiated T-cells which has been related to immunosenescence [10]. In addition, CMV may induce an adaptive differentiation and persistent expansion of an NKG2C<sup>+</sup> natural killer (NK)-cell subset [11], and this effect has been recently associated with decreased MS progression [12]. Herpesvirus coinfections might have additional immunological implications influencing the responses against other pathogens [13-15]. In this study we evaluated whether CMV infection and its imprint on the T- and NK-cell compartments may differ in early MS as compared to non-early MS patients, analyzing the putative association of this virus with MS clinical course and humoral immune responses against EBV and HHV-6.

## **METHODS**

### **MS patients and controls**

A cross-sectional multicenter study was performed in three hospitals integrated in the Spanish Network for MS Research (REEM) where MS patients were prospectively recruited from routine clinical visits. Patients (n=310) met MS criteria based on McDonald 2010. Exclusion criteria were (1) a relapse within 30 days prior to inclusion, (2) any severe concomitant disease, (3) pregnancy, and (4) disease-modifying drug (DMD) therapy known to deplete peripheral lymphocytes or alter their trafficking (i.e., fingolimod, natalizumab). Healthy controls (n=155) were included from the same geographic area than patients. Clinical variables (Table 1) were evaluated at the time of the analysis, including DMD treatment (interferon-beta, n=80; glatiramer acetate, n=8). Early MS (n=127, median time from onset: 1.33 years) was defined as a disease duration  $\leq 5$  years from the first clinical demyelinating event, classifying the remaining patients as non-early MS (n=183, median time from onset: 16 years). Blood samples from venous puncture were obtained for serological and immunological analysis. The study was approved by the IMIM Ethics Committee, including patients after written informed consent.

### **Immunological analysis of peripheral blood lymphocyte subsets**

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples taken in EDTA collection tubes using Lymphoprep, and subsequently cryopreserved in fetal calf serum with 10% DMSO. The gating strategy of the study is shown in Figure 1. NK-cell immunophenotype was evaluated in early MS patients (n=120) as previously reported [12], using the following conjugated monoclonal antibodies: anti-CD3-PerCP, anti-

CD56-APC (BD Biosciences), NKG2C-PE (R&D Systems), and DAPI. A selected panel of immunological markers of differentiated /senescent T-cells previously related to CMV infection (CD27-CD28-/CD57+/LILRB1+ T-cells) [16-18] were evaluated in a subcohort of MS patients (early MS, n=35; non-early MS, n=32) and controls (n=32) stratified for CMV serology. Samples were stained by indirect immunofluorescence with LILRB1 HP-F1 and anti-mouse Ig-PE-Cy7 (Biolegend), washed and further incubated with anti-CD3-APC-H7, anti-CD27-PERCP-Cy5.5, anti-CD28-PE-CF594, CD57-PE (BD Biosciences), and anti-CD4-FITC (eBioscience), anti-CD8-BV510 (Biolegend). All samples were analyzed at the Flow Cytometry Unit (UPF/CRG, Barcelona) with an LSRII-Fortessa flow cytometer (BD Biosciences).

### **Analysis of humoral responses to herpesviruses**

Standard clinical diagnostic tests to evaluate CMV and EBV specific circulating antibodies were performed in serum samples (BioMérieux). Enzyme-Linked ImmunoSorbent Assay (ELISA) tests were performed in subcohorts of MS patients (early MS, n=59; non-early MS, n=50) and controls (n=101) for quantitative determination of anti-EBNA-1 IgG and anti-VCA IgG (Trinity Biotech), anti-CMV IgG and IgM (Vircell), and anti-HHV-6 A/B IgG and IgM (Vidia) with commercially available kits following the manufacturer instructions. Results were expressed as an index value that was calculated as follows:  $[10 \times (\text{sample absorbance}/\text{cut-off value})]$ . Samples were analyzed in duplicate and doubtful samples (index value 9-11) were reanalyzed.

## Statistical analysis

After assessment of normal distribution using normal Q-Q probability plots, continuous variables were expressed as mean  $\pm$  standard deviation (SD) or median (first-third quartile) for parametric and non-parametric variables, respectively. Relationship between continuous and dichotomous variables was analyzed by Student t-test or Mann-Whitney U-test, respectively. Pearson or Spearman correlation indexes were calculated for pair-wise continuous variables. For a categorical analysis of NKG2C expression, a cut-off value was established at the third tertile of the distribution of the percentage of NKG2C+ NK-cells in healthy controls, classifying patients as low %NKG2C+ NK-cells (<19.3%) and high %NKG2C+ NK-cells ( $\geq$ 19.3%), as previously reported [12]. Logistic regression analysis determined predictors of CMV seropositivity adjusting the model for age and MS duration. Results were considered significant at the two-sided level of 0.05.

## RESULTS

### CMV seroprevalence in early MS patients

We first analyzed serum anti-CMV levels in MS patients (127 early MS, mean age,  $35 \pm 10.2$  years; 183 non-early MS,  $49.2 \pm 11.7$  years) and controls ( $n=155$ ,  $37.6 \pm 12.0$  years, aged-matched with early MS patients). Elevated anti-CMV IgM antibodies suggestive of recent asymptomatic primary infection were detected in 3 early MS patients. CMV seroprevalence did not differ in the whole cohort of MS patients as compared to controls (58.4% vs. 63.8%, respectively,  $p=0.149$ ) (Figure 2A). However, CMV seroprevalence in early MS (48%) was significantly lower compared two by two with non-early MS patients (65%,  $p<0.01$ ) and controls (63.8%,  $p<0.01$ ) (Figure 2B). Subsequently, cases were age-categorized in  $\leq 40$  or  $>40$  years-old. CMV seroprevalence was significantly lower in cases  $\leq 40$  years as compared to cases  $>40$  years, both in MS patients ( $p<0.001$ ) and controls ( $p<0.05$ ) (Figure 2C). MS patients  $\leq 40$  years had a significant lower CMV seroprevalence as compared to controls  $\leq 40$  years (45% vs. 67%,  $p<0.05$ ), but without differences between patients and controls older than 40 years (Figure 2C). No other clinical differences were associated with CMV seroprevalence in early MS (Table 1). A logistic regression analysis of the whole MS cohort showed that CMV seroprevalence was significantly associated with disease duration (odds ratio (OR) 1.04, 95% confidence interval (CI) 1.01-1.08,  $p<0.05$ ), independently of age (OR 1.02, 95% CI 0.99-1.04,  $p=0.190$ ).

### CMV-driven NKG2C<sup>+</sup> NK-cell expansion in early MS patients

We subsequently evaluated the CMV-imprint on the NK-cell compartment of early MS patients ( $n=120$ ). As previously reported (12), CMV seropositivity was associated with

higher %NKG2C<sup>+</sup> NK-cells in early MS patients (CMV<sup>-</sup>: 10.9% ± 5.7 vs. CMV<sup>+</sup>: 18.8% ± 16.5,  $p < 0.01$ ) and in controls (CMV<sup>-</sup>: 11.6% ± 8.2 vs. CMV<sup>+</sup>: 21.5% ± 19.2,  $p < 0.05$ ).

Classifying cases according to the magnitude of the NKG2C expansion, early MS patients with a high %NKG2C<sup>+</sup> NK-cells had lower disability scores as compared to patients with low %NKG2C<sup>+</sup> NK-cells (Table 1). No significant differences were found analyzing proportions of NKG2C<sup>+</sup> T-cells (early MS, 2.2% ± 2.0; non-early MS, 1.7% ± 2.0; controls, 1.8% ± 1.9,  $p = 0.187$ ). No additional differences were observed for CSF oligoclonal bands evaluated in a subcohort of 74 early MS patients (Supporting information, Table S1).

### **Association of CMV seropositivity with increased proportions of terminally differentiated T-cells in MS patients**

In order to evaluate the influence of CMV on the T-cell compartment, the expression of immunological markers of terminal differentiation was analyzed in a subcohort of MS patients (early MS,  $n = 35$ ; non-early MS,  $n = 32$ ) and controls ( $n = 32$ ). CMV seropositivity in patients and controls was associated with significantly higher proportions of CD27<sup>-</sup>CD28<sup>-</sup>, CD57<sup>+</sup> and LILRB1<sup>+</sup> in CD8<sup>+</sup> T-cells (Figure 3A), independently of age and MS duration. Regarding CD4<sup>+</sup> T-cells, CMV induced a less pronounced terminal differentiation than in CD8<sup>+</sup> T-cells, and was mainly observed in early MS and controls as compared to non-early MS (Figure 3B). Controls and non-early MS patients showed an age-related increase in differentiated CD8<sup>+</sup> T-cells, without additional differences observed according to DMD therapy (Supporting information, Table S2) or other clinical characteristics (data not shown).

### **Humoral immune responses to herpesviruses in early MS**

Virus-specific antibodies against CMV, EBV and HHV-6 were evaluated in early MS (n=59, 8 DMD-treated cases), non-early MS (n=50, 18 DMD-treated cases), and controls (n=101). Seroprevalences for herpesviruses in early MS compared to controls were as follows: CMV+, 44.1% vs.63% ( $p<0.05$ ); EBV+, 98.3% vs.88.5% ( $p<0.05$ ); and HHV-6+, 87.9% vs.89% ( $p=0.515$ ). Evaluating virus-specific quantitative indexes in seropositive individuals, MS patients had a significantly higher EBNA-1 index as compared to controls ( $24.25 \pm 4.96$  vs.  $18.93 \pm 4.06$ ,  $p<0.0001$ ) independently of MS duration (Figure 4A). Anti-VCA index was similar in early MS patients and controls (data not shown). No differences were observed for CMV or HHV-6 indexes according to MS duration (Figure 4B-C).

The putative influence of CMV infection with humoral responses to other herpesviruses was subsequently studied in individuals co-infected with EBV and HHV-6 (30 early MS, 23 non-early MS, 52 controls). CMV serostatus had no relationship with EBNA-1 or HHV-6 indexes in MS patients and controls (Figure 4D-E). However, an inverse correlation between anti-EBNA-1 and MS duration was observed in CMV+ MS patients ( $R_{\text{Spearman}} -0.59$ ,  $p<0.01$ ) but not in CMV seronegative cases (Figure 5), suggesting a progressive reduction in EBV humoral responses after MS onset in CMV-infected patients. No additional differences were detected regarding proportions of NKG2C+ NK-cells or DMD therapy (data not shown).

CMV IgG index was directly correlated with some markers of terminal differentiation in CD4+ and CD8+ T-cells in CMV+ MS patients and controls (Supporting information, Table S3). EBV humoral responses neither were related to

NKG2C<sup>+</sup> NK-cells expansion nor terminally differentiated T-cells, except for and inverse correlation of EBNA-1 index in early MS with percentages of CD3<sup>+</sup>CD56<sup>+</sup> ( $R_{\text{Spearman}} -0.55$ ,  $p < 0.01$ ), independently of CMV and HHV-6 serostatus. No correlations between EBV and HHV-6 serostatus and T-cell differentiation markers were found in MS patients or controls (data not shown).

## DISCUSSION

Infections have been considered as potential environmental factors involved in MS pathogenesis. EBV is the main pathogen suspected to be related to MS, suggesting the implication of virus-triggered immunological responses [1,9]. In contrast, some recent evidences support an inverse association between CMV and MS risk in pediatric and adult populations [3-5]. Our study shows a low CMV seroprevalence rate in early MS independently of age at disease onset, pointing out to epidemiological differences related to previous herpesvirus primary infections at early stages of the disease, in line with a putative protective role of CMV in MS.

Globally, CMV seroprevalence varies from an almost universal infection in undeveloped countries to 60-80% in western populations. CMV is transmitted through secretions and primary infection often occurs at early childhood. In this regard, we did not find serological evidences of CMV primary infection in early MS, except for 3 cases. Differences in seroprevalence for herpesviruses found in early MS might be related to the “hygiene hypothesis”, which proposes that early exposure in life to certain pathogens may be protective against autoimmune diseases, as opposed to a higher risk conferred by late primary infections [1]. The low CMV seroprevalence observed in early MS might be interpreted according to the “old friends” reformulation of the “hygiene hypothesis”, which attributes the increase in autoimmune disorders related to western lifestyle to a loss of symbiotic relationships with microorganisms that have a long-lasting co-evolution with humans [19].

Persistent CMV infection may have functional implications in the immune system, with a potential impact on the development of autoimmune diseases [20]. CMV develops a variety of immunoevasion strategies leading to complex host-virus

interactions. Expansions of oligoclonal CMV-specific effector-memory T-cell populations are detected in CMV+ individuals and tend to increase with aging [10]. Such “memory inflation” reflects an accumulation of oligoclonal differentiated T-cells, which have a limited proliferative capacity but maintain their effector functions upon antigen stimulation [16,18]. The reduced diversity of the CD8+ T-cell compartment has been related to immunosenescence and potentially limits the development of immune responses to unrelated antigens [10,18,21]. Based on our results, increased proportions of terminally differentiated T-cells were observed in CMV+ MS patients. Presumably, CMV seronegative MS patients close to disease onset develop the inflammatory process involving a pool of less differentiated T-cells as compared to CMV+ individuals. In this setting, persistent CMV infection might divert immunological resources reducing the risk of autoimmunity, in line with the hypothesis that it may be protective for MS development [4,5].

On the other hand, CMV infection may also have a positive indirect effect in the control of other viral infections. Previous exposure to some pathogens may modify specific immune responses to unrelated pathogens, a phenomenon termed “heterologous immunity”, which can be mediated by innate responses as well as cross-reactive T-cells, bystander activation of T-cells, and humoral responses [14,15]. Chronic infections may modify specific immunity against other pathogens impairing the development of CD8+ T-cell memory responses and inducing contraction of specific T-cell populations [22,23]. In particular, CMV may increase the production of proinflammatory cytokines (e.g., TNF- $\alpha$  and IFN- $\gamma$ ) and antibody-dependent cell-cytotoxicity mediated by adaptive NK-cells, which may influence immune responses to other pathogens [13,24]. On this basis, we analyzed specific humoral responses in MS patients according to CMV serostatus, observing a decrease in EBNA-1 index related to disease duration in CMV+

early MS patients. In contrast, no CMV-related differences were found when HHV-6 humoral responses were analyzed. Since anti-EBNA-1 antibody levels were previously shown to directly correlate with higher MS disease activity [1,8] and EBV-specific CD8<sup>+</sup> T-cells [7], further studies are needed to evaluate whether this CMV-related reduction in anti-EBNA-1 levels could be associated with decreased virus-specific T-cell responses.

In contrast to several evidences supporting a protective role of CMV in MS [3-5,12], other reports suggest a higher MS risk induced by this virus [6,25], an effect that could be related to the detrimental effect of CMV primary infection by increasing the inflammatory process in MS [6,25]. Nevertheless, our observations are consistent with a putative influence of a non-primary CMV infection in early MS. We did not find any significant clinical differences in early MS according to CMV serostatus. However, based on our results, lower disability scores at early stages of MS were associated with NKG2C<sup>+</sup> NK-cell expansion, in accordance with a previous study describing a lower risk of long-term progression in MS patients displaying such an NK-cell adaptive CMV-imprint [12].

In conclusion, early MS patients in our study were characterized by low CMV seroprevalence, suggesting that disease duration may be a relevant variable in studies analyzing the influence of this virus on MS risk. It is tempting to speculate that missing a potential “old friend” such as CMV could be associated to increased susceptibility for autoimmunity. By contrast, exposure to CMV in early childhood and the imprint of the virus on the immune system might influence the development of immune responses to other herpesviruses infections and decrease the risk of MS. The putative protective role conferred by CMV at early stages of MS remains to be addressed in further studies.

**Figure 1.** Flow cytometry immunophenotyping of CD3-CD56+ NK-cells and CD3+ T-cells. Representative examples of NKG2C+ NK-cell distributions are shown.

Terminally differentiated T-cell subsets were defined as CD27-CD28-, CD57+ and LILRB1+ CD8+ and CD4+ lymphocytes.

**Figure 2.** CMV serostatus in MS patients and controls. CMV seroprevalence in MS patients and controls (A), according to MS duration (early MS:  $\leq 5$  years) (B), and categorizing cases according to age  $\leq 40$  and  $> 40$  years-old (C). P-values: \* $< 0.05$ ; \*\* $< 0.01$ ; \*\*\* $< 0.001$ .

**Figure 3.** Terminally differentiated T-cells in MS patients and controls according to CMV serostatus and MS duration. Percentages of CD27-CD28-, CD57+ and LILRB1+ were evaluated in CD8+ T-cells (A) and CD4+ T-cells (B). P-values: \* $< 0.05$ ; \*\* $< 0.01$ ; \*\*\* $< 0.001$ .

**Figure 4.** Humoral immune responses to CMV, EBV and HHV-6 in MS patients and controls. IgG EBNA-1 index (A), IgG CMV index (B) and IgG HHV-6 index in MS patients and controls. EBNA-1 index in MS patients and controls according to CMV serostatus (D). IgG HHV-6 index in MS patients and controls according to CMV serostatus (E). P-values: \*\*\* $< 0.001$ ; \*\*\*\* $< 0.0001$ .

**Figure 5.** Correlations between EBNA-1 index and MS duration in early MS patients according to CMV serostatus.

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