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Intrathecal CD8 T-cells of multiple sclerosis patients recognize lytic Epstein-Barr virus proteins

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Abstract

Background: The association between Epstein-Barr virus (EBV) and multiple sclerosis (MS) may involve intrathecal EBV-specific T-cell responses targeting the virus or indirectly, autoantigens.

Objective: Compare the prevalence and fine-specificity of EBV-specific T-cells in the cerebrospinal fluid (CSF) of patients with MS ($n = 12$), clinically-isolated syndrome (CIS) ($n = 17$) and other neurological diseases (OND) ($n = 13$).

Methods: Intrathecal EBV-specific T-cell reactivity was assayed using CSF-derived T-cell lines (CSF-TCL) and autologous EBV-transformed B-cells (autoBLCL) as antigen-presenting cells (APC). EBV proteins recognized by autoBLCL-specific CD8 T-cells were identified using human leukocyte antigen class I (HLA-I)-negative monkey cells as artificial APC, co-transfected with 59 different EBV genes and the corresponding patient's HLA-I alleles that were involved in autoBLCL T-cell reactivity. Reactivity towards the MS-associated autoantigen αB-crystallin (CRYAB) was determined analogously.

Results: CSF-TCL from CIS and MS patients had significantly higher frequencies of autoBLCL-reactive CD4 T-cells, compared to the OND patients. CIS patients also had significantly higher autoBLCL-reactive CD8 T cells, which correlated with reactive CD4 T-cell frequencies. AutoBLCL-specific CD8 T-cell responses of four CSF-TCL analyzed in detail were oligoclonal and directed to lytic EBV proteins, but not CRYAB endogenously expressed by autoBLCL.

Conclusions: Enhanced intrathecal autoBLCL-specific T-cell reactivity, selectively directed towards lytic EBV proteins in two CSF-TCL, suggested a localized T-cell response to EBV in patients with MS. Our data warrant further characterization of the magnitude and breadth of intrathecal EBV-specific T-cell responses in larger patient cohorts.

Keywords: Autoantibodies, cerebrospinal fluid, clinically-isolated syndrome, crystallin, disease control, Epstein-Barr virus, herpesviridae, intrathecal space, lytic antigen, multiple sclerosis, T-cells, target antigen

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Introduction

Multiple sclerosis (MS) is characterized by the development of foci of demyelinating and axon-damaging inflammation that spreads throughout the central nervous system (CNS). Clinically-isolated syndrome (CIS) may precede definitive MS. The clinical course of MS may follow a variable pattern over time and MS is classified into three categories: relapsing–remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS).[1] Although the clinical course of MS is highly variable, most patients eventually develop severe neurological disability.

The cause and pathogenic mechanisms of MS remain enigmatic. MS is considered the result of a local inflammatory response, involving both resident cells (e.g. microglia) and infiltrating T-cells in the CNS, in

genetically susceptible individuals; initiated by environmental factors, most likely viruses.[1,2] Recent studies advocate the role of Epstein-Barr virus (EBV) during the initiation and potentially the perpetuation of MS pathogenesis.[2] Indicative of this are both higher EBV seroprevalence and systemic virus-specific B- and T-cell responses in MS patients, compared to controls[3–5]; the epidemiological relationship between infectious mononucleosis and MS[6]; the presence of EBV-infected B-cells in MS lesions and viral deoxyribonucleic acid (DNA) in cerebrospinal fluid (CSF).[7–9] However, evidence for the presence of EBV in the lesions and CSF of MS patients remain controversial.[10–12]

EBV is a human B-cell tropic γ-herpesvirus that is endemic worldwide.[13] The virus' hallmark is to establish a lifelong latent infection in B-cells. The T-cells are pivotal in controlling viral latency.[13,14] EBV may evoke T-cell-mediated MS pathology in different ways. Virus-specific T-cells may recognize EBV-infected cells in the brain,[7,15] cross-react with myelin antigens,[16,17] or recognize autoantigens (e.g. αB-crystallin (CRYAB)) induced in B-cells upon EBV infection.[18] Given the limited access to brain tissue, studies on local antigen-specific T-cell responses in MS patients are restricted to CSF samples. CD4 and CD8 T-cells that are reactive to autologous EBV-transformed B-cell lines (BLCL), in part directed to EBV nuclear antigen 1 (EBNA-1), can be cultured from CSF from MS patients.[17,19,20] Recently, two analogous studies on larger patient cohorts, including MS patients and disease controls, compared EBV- and cytomegalovirus (CMV)-specific T-cell responses in paired blood and CSF.[21,22] Whereas both groups describe a selective increase of intrathecal EBV-specific, but not CMV-specific T-cells, the EBV T-cell responses were either restricted to CD8 T-cells mainly in patients with early MS[21] or limited to CD4 T-cells in RRMS patients.[22] Furthermore, deep sequencing of T-cell receptor β (TCRBV) gene usage in paired blood and CSF samples confirm intrathecal enrichment of EBV-specific CD8 T-cells in MS[23]. However, the latter studies did not completely determine the viral proteins recognized or were limited to an analysis of a selected set of known immunodominant EBV CD8 T-cell epitopes.[19–22]

The aim of the current study was to determine the prevalence and fine specificity of EBV-specific T-cells cultured from the CSF of CIS and MS cases, and patients with other neurological disease (OND). A schematic overview of the subsequent experimental procedures performed to address these important issues in MS pathology is presented in Figure 1.

Materials and methods

Patients and clinical specimens

Between December 2008 and June 2012, paired blood and CSF samples from CIS (n = 17), MS (n = 12) and OND patients (n = 5) were obtained as part of a diagnostic workup at the Erasmus Medical Center (Rotterdam, the Netherlands). Informed consent was obtained from each patient. The study was approved by the local ethical committee and performed according to the tenets of the Helsinki declaration. The MS patient cohort consisted of RRMS (n = 10) and PPMS (n = 2) and CIS (n = 17) patients, according to the 2010 Revised McDonald Criteria. The OND cohort consisted mainly of paraneoplastic neurological syndrome patients (n = 9) with high serum Human neuroantigen D (HuD)-specific antibody titers; and OND patients (n = 4), whom suffered from a variety of clinical symptoms, including headache and subarachnoid hemorrhage, all sampled between February 2005 and September 2007.[24] No patient was receiving corticosteroids nor immunomodulatory therapy at the time of their lumbar puncture, which was always performed > 1 month after the clinical symptoms began. We typed the HLA Class I (HLA-I) genotype of specific patients, using standard diagnostic polymerase chain reaction (PCR) at a 4-digit resolution level.

EBV serology and real-time PCR

The total IgG levels, and the IgG levels directed to EBV virus capsid antigen (VCA) and EBNA-1, were determined from the serum, using chemoluminescent assays on a Liaison XL analyzer (Diasorin, Torino, Italy), as per the manufacturer's instructions. The EBV-specific intrathecal IgG production was determined for EBNA-1 by calculating the Golmann-Witmer coefficient (GWC), as described previously.[25] A GWC > 3 was considered indicative for intrathecal EBV-specific IgG production. We determined the presence of EBV DNA in the CSF, and human CRYAB and β-actin transcript levels in BLCL, by real-time PCR (qPCR) and reverse-transcribed qPCR on an ABI Prism 7700 with Taqman Universal Master Mix and the custom-made (EBV gene BNRF1)[26] or commercial intron-spanning primer/probe-pairs specific for human CRYAB and β-actin (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's instructions, respectively.

We determined the relative transcript levels by the formula (Equation 1):

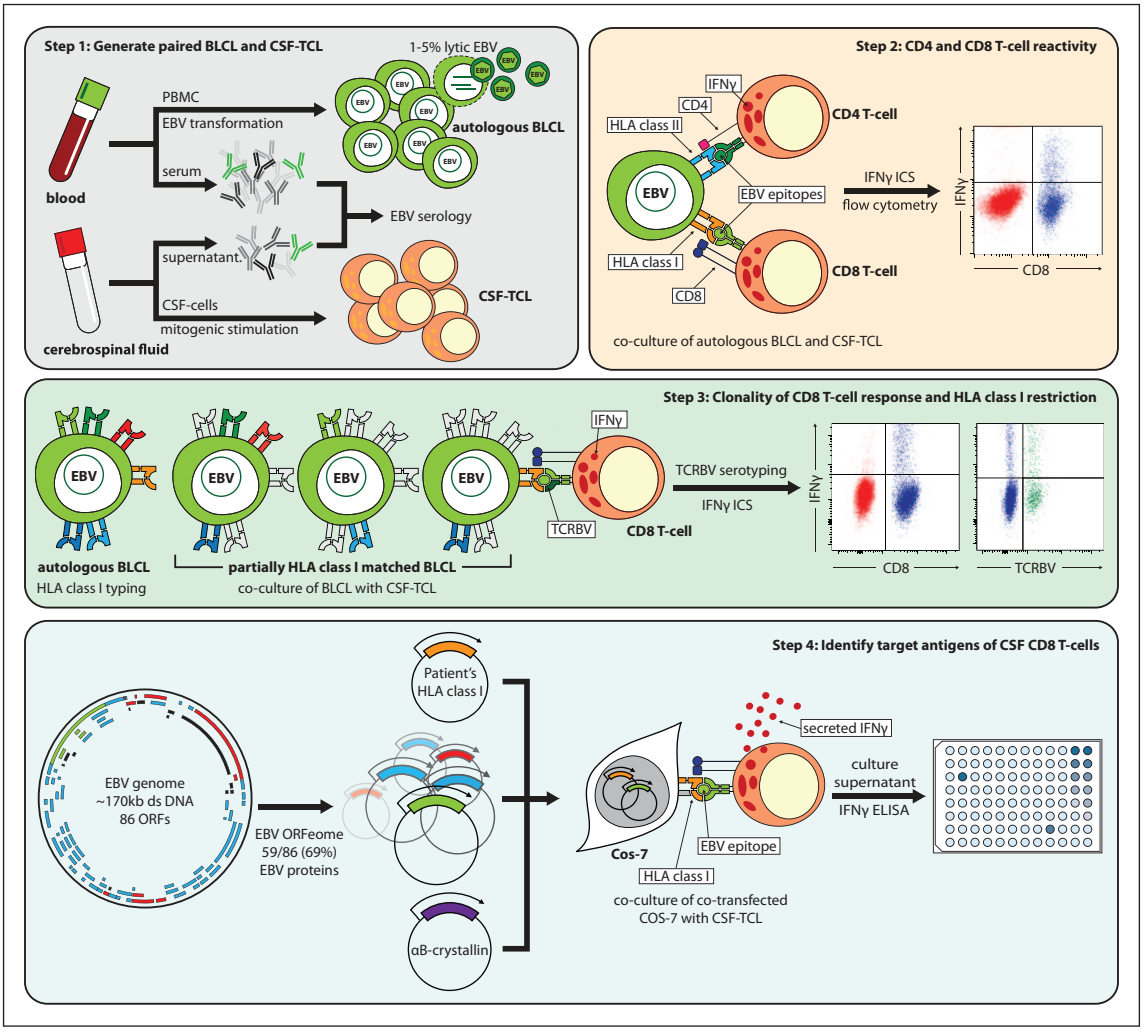

Figure 1. Schematic overview of experimental procedure to identify intrathecal EBV-response. We generated paired EBV-transformed BLCL and CSF-TCL. Co-culture of CSF-TCL with the autologous BLCL as APC; followed by CD3, CD4, CD8, ICS IFN-γ and flow cytometry allowed simultaneous analysis of CD4 and CD8 T-cell reactivity. Subsequently, the clonality and HLA-I restriction of BLCL-specific CD8 T-cells was assayed by co-culture of CSF-TCL with partially HLA-I-matched allogeneic BLCL, followed by combined IFN-γ ICS and T-cell receptor Vβ analysis. Next, EBV targets of BLCL-reactive CD8 T-cells were identified. Therefore, an EBV ORF library encoding 59 of the 86 EBV proteins and EBV-induced CRYAB was cloned in expression vectors. HLA-I-deficient Cos-7 cells were co-transfected with expression vectors encoding the EBV ORFeome or CRYAB and the previously-identified patients' HLA-I and used as the artificial APC in a co-culture with CSF-TCL. The conditioned culture supernatants were screened for secreted IFN-γ using ELISA, for a qualitative identification of the CD8 T-cell targets.
APC: antigen-presenting cells; BLCL: blood-derived EBV-transformed B cell lines; CRYAB: alpha-B-crystallin; CSF-TCL: CSF-derived T-cell lines; EBV: Epstein-Barr virus; ELISA: enzyme-linked immunosorbent assay; ICS: intracellular staining; IFN: interferon; HLA-I: human leukocyte antigen Class I; ORF: open reading frame

$$10,000 \times 2^{(-\text{delCt})} \quad (1)$$

Here (Equation 2):

$$\text{delCt} = \text{Ct}((\text{CRYAB}) - \text{Ct}(\beta - \text{actin})) \quad (2)$$

Generation of BLCL and CSF-TCL

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation of heparinized peripheral blood and the B-cells were infected with EBV strain B95.8 to generate BLCL, as described previously.[27] The CSF-derived T-cell lines (CSF-TCL) were

Table 1. General characteristics of the study population analyzed for EBV reactivity.

Neurological disease entity	*N*	Gender *n* (M:F)	Median age (yrs (IQR))[a]	Median disease duration (mos (IQR))[a]
MS patients	29	10 : 19	38 (34–41)	7 (2–22)
Relapsing–remitting MS	10	2 : 8	37 (33–39)	16 (3–69)
Primary progressive	2	1 : 1	51 and 55[b]	10 and 54[b]
Clinically-isolated syndrome	17	7 : 10	38 (34–41)	4 (1.5–10)
Disease controls[c]	13	7 : 6	66 (54–70)	3 (2–8)
HuD-PNS	9	3 : 6	66 (54–70)	3 (3–8)
Miscellaneous[d]	4	4 : 0	64 (57–72)	1 (0.08–8)

[a]All values are as median (IQR).
[b]The age and disease duration of two patients with primary progressive MS are presented.
[c]HuD-PNS: PNS patients with high serum HuD-specific antibody titers.
[d]Miscellaneous: Patients with variety of clinical symptoms including headache and subarachnoid hemorrhage.
EBV: Epstein-Barr virus; F: female; HuD: Human neuroantigen D; IQR: interquartile range; M: male; MS: multiple sclerosis; PNS: paraneoplastic syndrome; yrs: years.

obtained by nonspecific stimulation of cells from CSF with 1 μg/ml phytohemagglutinin-L (PHA-L) (Roche, Branford, CT, USA) in the presence of γ-irradiated allogeneic PBMC (3000 rad; 10^5/well) in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated pooled human serum (Sanquin, Amsterdam, the Netherlands) and 50 IU/ml recombinant interleukin-2 (IL-2) (Miltenyi, Bergisch Gladbach, Germany); and then cultured for about 2 weeks at 37°C.[24,27] The CSF-TCL with insufficient cell numbers (i.e. < 5 x 10^6) were restimulated with an anti-CD3, from clone OKT3 (Janssen-Cilag, Tilburg, the Netherlands).[28] At the time of inclusion, the co-authors who generated the CSF-TCL and autologous BLCL (autoBLCL) were unaware of the patients' clinical diagnosis. Identical protocols were used previously, to generate paired CSF-TCL and autoBLCL from HuD-PNS patients at the same lab (Department of Viroscience, Erasmus Medical Center, the Netherlands), under supervision of the senior author.[27] For the latter patient group, the researchers were aware of their clinical diagnosis beforehand. Notably, of the 47 paired peripheral blood and surplus CSF samples obtained between December 2008 and June 2012, the generation of BLCL and CSF-TCL with sufficiently high T-cell numbers was unsuccessful for five and 13 patients, respectively. We obtained successful CSF-TCL to high numbers from all the MS patients, but only in 17 of 28 (61%) CIS, and 3 of 5 (60%) of the OND patients. Overall, the paired BLCL and CSF-TCL of 29 MS patients and 13 OND patients were available to study intrathecal EBV-specific B- and T-cell reactivity (Table 1).

T-cell reactivity to autologous BLCL

EBV reactivity of CSF-TCL was determined by intracellular staining (ICS) for interferon γ (IFN-γ) using autologous BLCL (autoBLCL) as antigen presenting cells (APC).[28] As a positive control for T-cell activation, CSF-TCL were stimulated for 6 hours with a combination referred to as P/I, of phorbolmyristate-acetate (PMA) at 50 ng/ml and ionomycin at 500 ng/ml (Sigma, St Louis, MO, USA).[27,28] T-cells were phenotyped with fluorochrome-conjugated monoclonal antibody (mAb) directed to human CD3 (clone SP34-2; BD Pharmingen, San Diego, CA, USA), CD4 (clone SK3; BD Biosciences, San Jose, CA, USA), CD8α (clone RPA-T8; eBiosciences, San Diego, CA, USA) and IFN-γ (clone B27, BD Pharmingen, San Diego, CA, USA) and stained for viability (violet live/dead stain; Invitrogen); and then analyzed using a FACSCanto flow cytometer and CellQuest Pro software (BD Biosciences), as described.[27,28]

Experiments were performed at least two times and as negative controls, the appropriate isotype- and fluorochrome-matched unrelated mAbs were included. Except for the HuD-PNS patient cohort, the team was blinded for the patients' clinical diagnosis during autoBLCL T-cell reactivity analyses. All subsequent in-depth T-cell analyses were performed on CSF-TCL of selected high autoBLCL responding CIS and MS patients. Enumeration of activated T-cells, based on their intracellular IFNγ expression, was defined by gating on the viable cells that expressed surface CD3, CD4 or CD8. For all assays, at least 5 x 10^3 gated viable CD4 and CD8 T-cells were obtained; and the threshold gate for autoBLCL-reactive T-cells was set at 0.1% IFN-γ^+ CD4 and CD8 T-cells, based on unstimulated CSF-TCL for each donor. All T-cell assays were performed and the data interpreted by the team, according to longstanding in-house standard operating procedures (SOPs) in the Department of Viroscience (Erasmus MC).

Identification of the HLA-I allele involved in CD8 T-cell reactivity to BLCL

Identification of the patient's HLA-I allele involved in autoBLCL CD8 T-cell reactivity was determined using partially HLA-I matched allogenic BLCL as the APC. Furthermore, CSF-TCL clonality was determined using a multiparametric analysis tool (IOTest Beta Mark mAb Kit; Beckman Coulter, Marseille, France), which contains multiple fluorochrome-conjugated mAbs directed to approximately 70% of all known TCRBV.[29] Combined extracellular TCRBV and intracellular IFN-γ staining facilitated the differentiation between BLCL and allo-HLA reactivity of the corresponding CSF-TCL by flow cytometry. Experiments were performed at least two times.

T-cell reactivity to the EBV proteome and human αB-crystallin

The generation and validation of individual expression vectors (cloned in the pCMV-EHis vector) containing the EBV proteome, covering a total of 59 of 85 (69%) of all known EBV proteins that were used as target antigens for CSF-TCL, have been detailed elsewhere.[30] Human CRYAB cDNA (Darmacon, Lafayette, CO, USA) was cloned in the custom-made eukaryotic pNS.CD8α vector.[31]

We determined the EBV proteome and CRYAB T-cell reactivity on artificial APC, consisting of Cos-7 monkey fibroblasts (American Type Culture Collection (ATCC), Manassas, VA, USA) that were co-transfected with the patient-matched HLA-I allele-specific cDNA (cloned in vector pcDNA3) and expression vectors encoding 59 individual EBV protein-encoding genes or human CRYAB, as described.[28] All co-transfections were performed in duplicate; and the appropriate negative (untransfected Cos-7 cells) and positive controls (autologous BLCL and P/I) were included. CSF-TCL were added to co-transfected Cos-7 cells; and then after 24 hrs, the culture supernatants were collected for IFN-γ ELISA.[28] Expression of specific EBV proteins (i.e. EBNA1 (clone OT1x), LMP1 (clone OT21c), BZLF1 (clone BZ1), BALF2 (clone OT9-2) and BMRF1 (clone OT14e-2)) by BLCL, and expression of human CRYAB (clone 2E8) (AbD Serotec, Oxford, UK) by co-transfected Cos-7 cells, was confirmed by conventional immunocytology on fixed cells, using fluorescein-conjugated goat-anti-mouse IgG (BD Biosciences). Experiments were performed at least two times.

Statistical analyses

Statistical differences were determined by the Mann-Whitney U test, or in the case of paired specimens, by Spearman's rank correlation test using Prism (GraphPad 5.0 Software, La Jolla, CA, USA).

Results

Intrathecal T-cell reactivity to autologous BLCL increased in CIS and MS patients

To study the role of EBV infection in MS, we analyzed virus-specific B- and T-cell responses in surplus CSF from CIS ($n = 17$), MS ($n = 12$) and OND patients ($n = 13$) (Table 1). Laboratory diagnostics on paired serum and CSF of the CIS and MS cases showed that all patients were EBNA-1 seropositive, except one CIS patient, whom was EBNA-1 seronegative but VCA IgG seropositive. Local antibody response to EBNA-1 and EBV DNA were undetectable in the CSF of all CIS and MS patients (data not shown). Paired surplus CSF and serum of OND patients were not available for retrospective EBV diagnostics.

To compare intrathecal EBV-specific T-cell responses between patient cohorts, we expanded the CSF T-cells non-specifically, with PHA-L and subsequent anti-CD3, to obtain high T-cell numbers (Figure 1). In contrast to CD4 T-cells, CD8 T-cell frequencies were significantly higher in the CSF-TCL of CIS patients (mean ± standard error (SE) = 31 ± 4; $p = 0.009$), compared to the MS (28 ± 6) and OND patients (16 ± 4). AutoBLCL were generated by EBV infection of blood-derived B cells. Concordant with earlier studies, the majority of BLCL cells expressed the viral proteins EBNA1 and LMP1, which represent latently-infected B cells.[13] Notably, about 2–5% of the BLCL cells had entered the lytic cycle, and most likely expressed the whole EBV proteome (Supplementary Figure 1). Accordingly, BLCL were considered appropriate APC to analyze intrathecal EBV-specific T-cell reactivity.[14,30]

To determine if our protocol to generate CSF-TCL from different patient cohorts, and in particular long-term storage of HuD-PNS cell lines in liquid nitrogen, influenced the functionality of CSF-derived T-cells, all CSF-TCL were assayed for IFNγ expression upon P/I stimulation. The average frequency of $IFN\gamma^+$ CD4 and CD8 T-cells in P/I-stimulated CSF-TCL was > 80% in all patient groups, indicating that the methodology used and long-term storage of CSF-TCL did not negatively affect T-cell immune competence of CSF-derived T-cells (Supplementary Figure 2). Subsequently, the CSF-TCL were assayed for autoBLCL reactivity.

Frequencies of autoBLCL-reactive CD4 T-cells were significantly higher in CSF-TCL of CIS (mean ± SE:

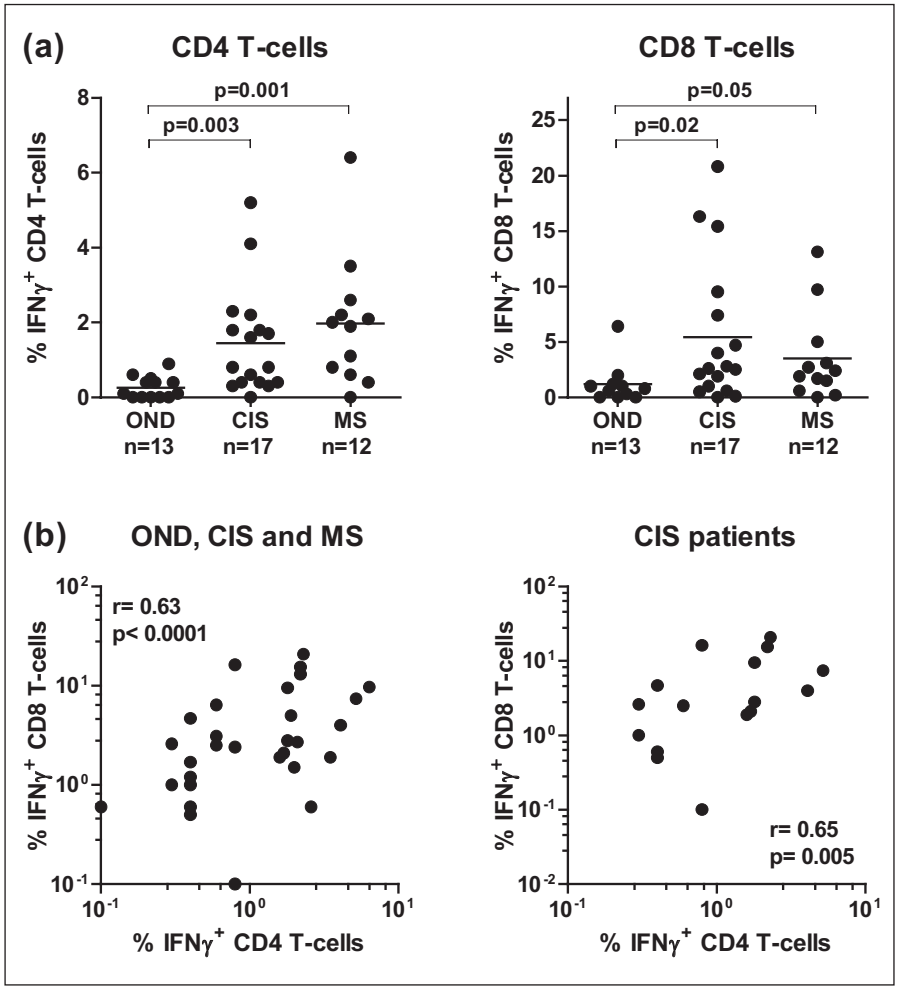

Figure 2. Increased intrathecal CD4 and CD8 T-cell reactivity to autologous BLCL, in CIS and MS patients. (A) T-cell lines generated from CSF of MS and CIS patients, and as a control, patients with OND; were incubated with autologous EBV-transformed B-cell lines. Phenotype and frequency of the reactive T-cells was determined by flow cytometry, using specific monoclonal antibodies directed to CD3, CD4, CD8 and intracellular IFN-γ. (B) The correlation of autoBLCL-reactive CD4 and CD8 T-cell frequencies of individual CSF-TCL of all three patient groups (left panel) or solely CIS patients: Dots represent data of individual CSF-TCL, presented as the percentage of IFN-γ^+ cells of all CD4 or CD8 T cells in the corresponding CSF-TCL, and the bars show the average values of each patient group. The statistical analyses used were the Mann-Whitney U test (A) and the Spearman's rank correlation test (B).
autoBLCL: autologous EBV-transformed B-cell lines; CSF: cerebrospinal fluid; CSF-TCL: T-cell lines generated from CSF; IFN: interferon; OND: other neurologic diseases

1.5 ± 0.3; $p = 0.003$) and MS patients (2.0 ± 0.5; $p = 0.001$), compared to disease controls (0.3 ± 0.1), as seen in Figure 2(a). For CD8 T-cells, CIS patients (5.4 ± 1.6; $p = 0.02$), but not MS patients (3.5 ± 1.2; $p = 0.05$) had significantly higher autoBLCL-reactive T-cell frequencies, compared to disease controls (1.2 ± 0.6). No significant differences were detected between MS and CIS patients (Figure 2(a)). Overall, frequencies of autoBLCL-reactive CD8 T-cells (3.7 ± 0.8) were significantly higher than in CD4 T-cells (1.2 ± 0.2; $p = 0.01$). Notably, intra-patient frequencies of CD4 and CD8 autoBLCL-reactive T-cells correlated significantly; which upon analysis of the three patient groups separately, was only significant for the CIS patients (Figure 2(b)).

Intrathecal CD8 T-cell reactivity to autologous BLCL is directed to lytic EBV proteins

Recent data argue for the potential role of CD8 T-cells in MS pathology.[1,2] Increased frequency of autoBLCL-reactive CD8 T cells, predominantly in CIS patients (Figure 2(a)), prompted us to determine if these CSF-derived CD8 T-cells recognized EBV proteins (Figure 1). EBV protein specificity was determined using a

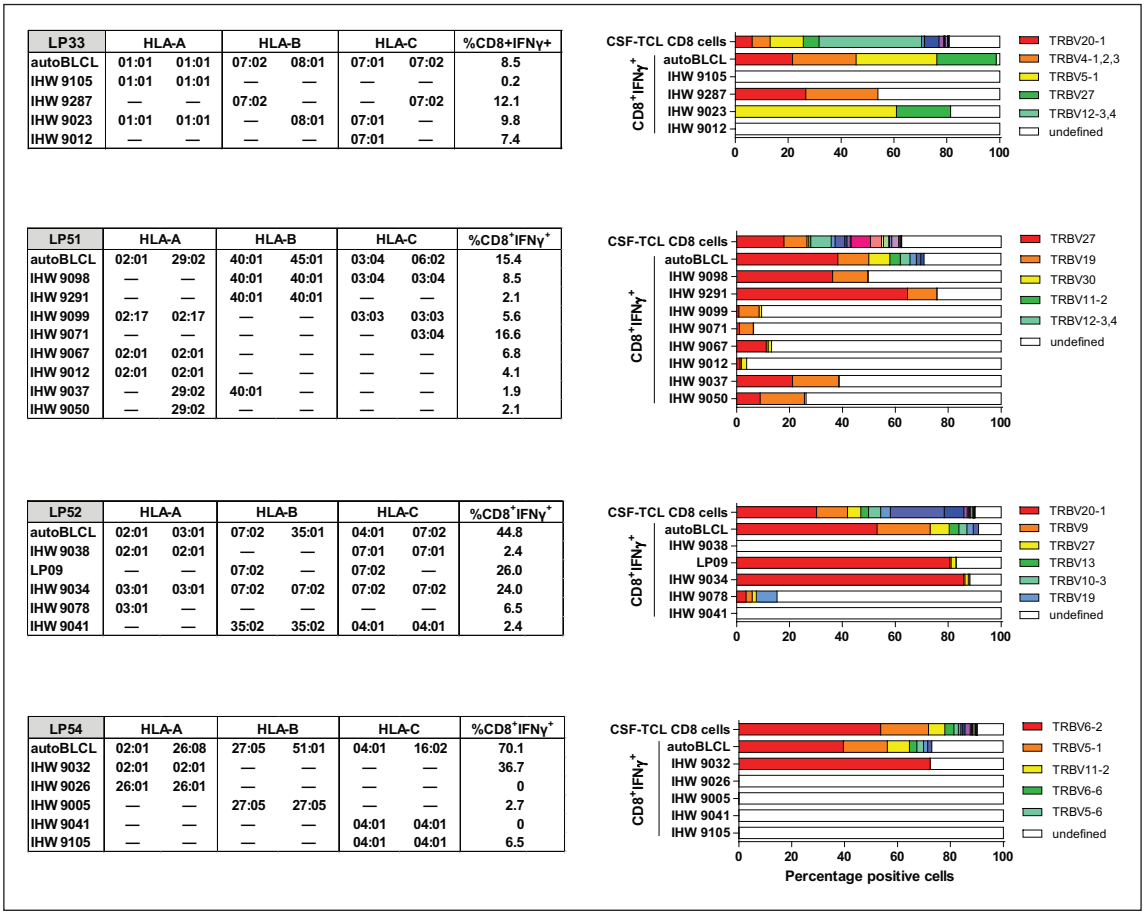

Figure 3. Identification of the patient's HLA-I allele involved in intrathecal CD8 T-cell reactivity to autologous BLCL. TCL generated from CSF (CSF-TCL) of three CIS patients (numbers LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated with autoBLCL and various partially-matched HLA-I allogeneic BLCL. The TCRBV gene usage of BLCL-reactive CD8 T-cells was determined by flow cytometry, using specific mAbs directed to different TCRBV (covering about 70% of all known TCRBV), CD8 and intracellular IFN-γ. The left panel shows the HLA-A, HLA-B and HLA-C alleles, expressed by the allogeneic BLCL, that match the patients' HLA-I genotype and the corresponding frequency of BLCL-reactive CD8 T cells, presented as the percentage of IFN-γ^+ cells of all the CD8 T-cells in the corresponding CSF-TCL (last column). The right panel shows the TCRBV gene usage of all CD8 T-cells in the CSF-TCL (upper row) and the autoBLCL-reactive CD8 T-cells (subsequent rows). For the latter analysis, the percentage of autoBLCL-reactive CD8 T-cells was set to 100%, to facilitate inter-BLCL comparison of the TCRBV gene usage of the BLCL-specific CD8 T-cells.

[a]Herein, undefined indicates that the corresponding TCRBV could not be determined with the TCRBV-specific mAbs used.

autoBLCL: autologous EBV-transformed B-cell lines; BLCL: blood-derived EBV-transformed B-cell lines; CIS: clinically-isolated syndrome; CSF: cerebrospinal fluid; EBV: Epstein-Barr virus; HLA-I: human leukocyte antigen Class One; mAbs: monoclonal antibodies; MS: multiple sclerosis; RRMS: relapsing–remitting MS; TCL: T-cell lines; TCRBV: T-cell receptor V beta

recently-developed viral proteome approach, using artificial APC, consisting of Cos-7 cells co-transfected with 59 individual EBV protein-encoding genes and the patient HLA-I alleles identified as the restriction element of the autoBLCL-specific CD8 T-cell response.[28] Because both high numbers of T-cells and relatively high frequencies of reactive CD8 T-cells (> 4%) are mandatory for definitive viral proteome screening hits, the EBV proteome approach was only possible for CSF-TCL of three CIS patients (LP33, LP52 and LP54) and one RRMS patient (LP51).[28]

First, we determined the patients' HLA-I alleles involved in the autoBLCL CD8 T-cell responses, by using a combined flow cytometry assay with partially HLA-I-matched BLCL as APC and TCRBV phenotyping, to differentiate between BLCL and allo-HLA-reactive T-cells (Figure 1 and Figure 3). Whereas all four CSF-TCL contained a polyclonal CD8 T-cell population, selective IFN-γ expression combined with restricted TCRBV usage indicated that CD8 T-cell reactivity was restricted by one (LP52: HLA-B*0702 and LP54: HLA-A*0201), two (LP33:

HLA-B*0702 and HLA-B*0801) and four (LP51: HLA-A*0201, HLA-A*2902, HLA-B*4001 and HLA-C*0304) HLA-I alleles. Our data implicate that the autoBLCL-reactive CD8 T-cell repertoire in all four CSF-TCL is oligoclonal (Figure 3). Finally, the CSF-TCL were subjected to the EBV proteome assay, using the identified HLA-I alleles (Figure 1). The EBV proteome collection contained 59 of 85 (69%) of all known EBV genes and covered viral proteins that are selectively expressed during latency and those co-expressed during lytic EBV infection; including immediate early, early and late viral proteins.[13,30] In total, four out of eight (50%) autoBLCL/HLA-I allele combinations tested revealed reproducible specific CD8 T-cell EBV protein hits (Figure 4).

CD8 T-cell recognition of autologous BLCL is not directed to αB-crystallin

The inability to identify the cognate EBV protein of the remaining four out of eight autoBLCL/HLA-I allele combinations may be because the EBV proteome collection was not complete: 26 of the 85 (31%) known EBV proteins are not included.[30] Alternatively, CD8 T-cells are directed to host cell antigens expressed by BLCL. The heat shock protein CRYAB is a potential candidate host protein, as this protein is identified as a T-cell autoantigen in MS patients, and EBV infection of B-cells induces CRYAB expression.[1,18] Indeed, readily-detectable levels of CRYAB transcript were detected in the BLCL of representative patients (Figure 5(a)). Next, we cloned and expressed human CRYAB in Cos-7 cells, along with the respective HLA-I alleles identified as the restriction element of the autoBLCL CD8 T-cell response (Figure 3). The CRYAB protein expression was detectable in transfected Cos-7 cells (Figure 5(b)). However, no CSF-TCL recognized Cos-7 cells transfected with the respective CRYAB/HLA-I combinations, indicating that the intrathecal autoBLCL CD8 T-cell response was not directed to CRYAB (Figure 5(c)).

Discussion

Despite strong epidemiological evidence, the potential pathological mechanisms initiated by EBV in MS remain unclear.[1,2,11] If EBV is involved then the virus' signature, either directly as viral remnants (e.g DNA) or indirectly as target of a local host immune response, should be selectively detectable or increased in brain tissue or alternatively CSF of MS patients. Most likely at early onset of disease.[1,2] Data presented here supported the involvement of intrathecal EBV-specific T-cell responses in MS, particularly in CIS patients. We detected significantly higher frequencies of autoBLCL-reactive CD4 T-cells in the CSF-TCL from both CIS and MS patients, compared to OND patients (Figure 2(a)). Moreover, CIS patients also had significantly higher autoBLCL CD8 T-cell reactivity, which only in this patient group was correlated with paired reactive CD4 T-cell frequencies. This suggested a coordinated intrathecal CD4 and CD8 T-cell response directed toward either EBV or host antigens, expressed by autoBLCL (Figure 2(b)).

Data are consistent with earlier reports on T-cell reactivity to autoBLCL in the CSF of MS patients.[17,19,20] In contrast, neither EBV DNA nor intrathecal virus-specific IgG production was detectable in the CSF of the CIS and MS patients (data not shown). Whereas the latter data concur with reports that refute the role of EBV in MS,[10–12,25,32] other groups did find EBV remnants and increased virus-specific IgG in the CSF of MS patients.[7–9,33,34] The relatively long time interval at which the CSF samples of our patient cohort were obtained (> 1 month after onset of disease) may have impeded detection of EBV DNA, but most likely not intrathecal EBV-specific IgG production.[12,14] This B-cell/T-cell discrepancy may be methodological.[2,3] Whereas intrathecal T-cell reactivity was performed on BLCL that in part expressed the whole EBV proteome, local antibody production was assayed only on one EBV protein (EBNA1). Alternatively, the autoBLCL T-cell reactivity was not directed to EBV proteins, but to host proteins like the MS-associated autoantigen CRYAB, endogenously expressed by BLCL.[2,18] Previous studies had not completely deciphered this autoBLCL phenomenon.[17,19,20] They were either restricted to the analysis of a selective set of known immunodominant EBV peptides or used autologous dendritic cells pulsed with EBV or BLCL protein lysates as APC.[19–22] Besides the limited number of peptides tested, which discounts issues like inter-HLA allele peptide affinity and post-translational peptide modifications, the caveat of the latter strategy is restriction to structural viral proteins and potential contamination of host proteins in case of EBV and BLCL protein lysates, respectively.[13,14,30]

The artificial APC system applied here overcomes these potential drawbacks, by assaying CD8 T-cell reactivity on cells that both endogenously express individual EBV proteins or human CRYAB, along with the HLA-I alleles that are involved in autoBLCL CD8 T-cell recognition.[28] Concurrent with a previous report no autoBLCL reactive T-cell recognized CRYAB.[18] Although BLCL predominantly contained B cells expressing latent viral proteins (Supplementary Figure 1), the intrathecal CD8 T-cell targets were

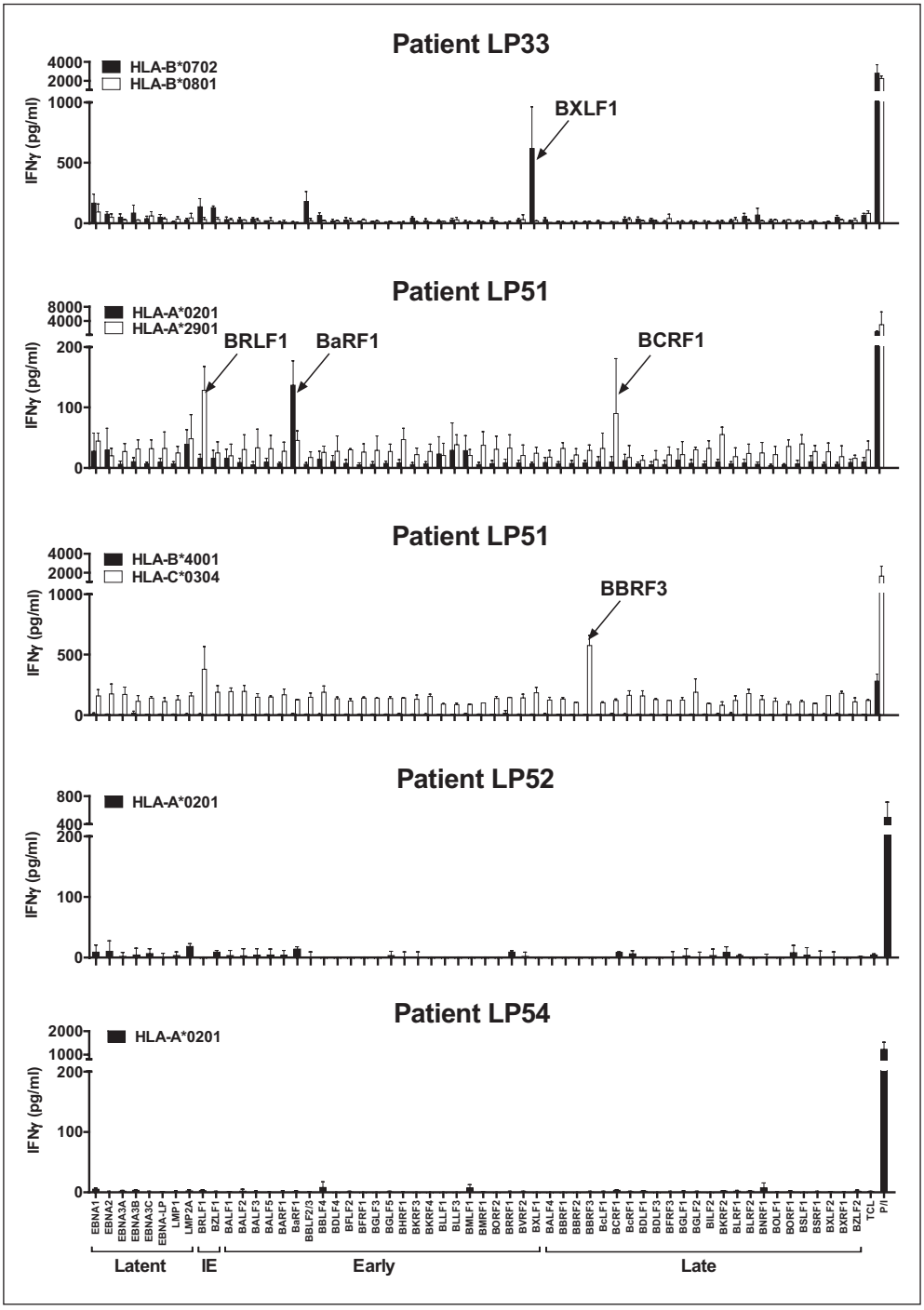

Figure 4. Intrathecal autoBLCL-reactive CD8 T cells recognize lytic EBV proteins. T-cell lines generated from CSF, of three CIS patients (numbers LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated, in duplicate, with Cos-7 cells that co-expressed the respective donor-specific HLA-I allele and individual EBV proteins ($n = 59$); and T-cell reactivity was measured by quantifying IFN-γ secretion in the cultures' supernatants. The EBV proteins are arrayed on their kinetic class level in a nominal genomic order on the x-axis and the IFN-γ secretion levels (mean ± SD) of 2–3 independent experiments are shown. The names of EBV proteins that are specifically recognized are indicated by an arrow. The results of the EBV proteome analyses on the CSF-TCL for individual HLA-I alleles of patients LP33 ($n = 2$) and LP51 ($n = 4$) are shown in black and grey values. The negative and positive controls were CSF-TCL incubated with untransfected Cos-7 cells or CSF-TCL stimulated with PMA and ionomycin, respectively.

autoBLCL: autologous EBV-transformed B-cell lines; CIS: clinically-isolated syndrome; CSF: cerebrospinal fluid; EBV: Epstein-Barr virus; IFN: interferon; MS: multiple sclerosis; P/I: PMA and ionomycin; PMA: phorbol-myristate acetate; RRMS: relapsing–remitting MS; TCL: T-cell lines

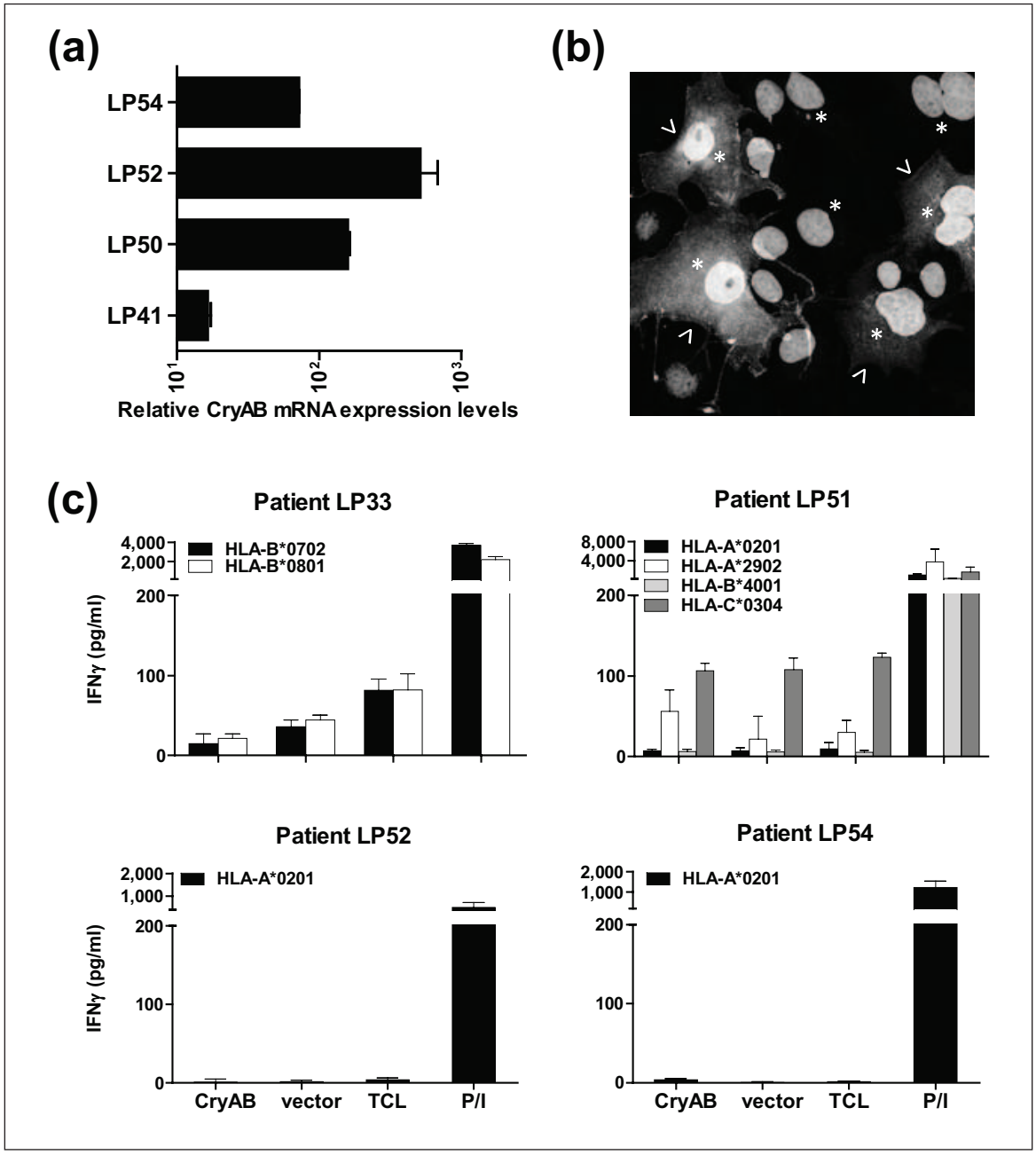

Figure 5. CD8 T-cell recognition of autologous BLCL is not directed to αB-crystallin. (a) Relative CRYAB transcript levels were determined by reverse transcriptase human CRYAB-specific real-time PCR on RNA isolated from BLCL from the indicated patients. Relative transcript levels were determined by the formula 10,000 x 2(-delCt), where delCt equals Ct ((CRYAB) - Ct (β-actin)). (b) The Cos-7 cells transfected with human CRYAB were assayed for CRYAB protein expression by immunocytology, using a specific mAb, and subsequently visualized using a fluorescein-conjugated secondary antibody (arrowheads). The nuclei were stained with DAPI (asterisks). Original magnification was 400x. (c), T-cell lines generated from CSF of three CIS patients (patients LP33, LP52 and LP54) and one RRMS patient (LP51) were incubated, in duplicate, with Cos-7 cells that co-expressed the respective donor-specific HLA-I allele and human CRYAB. T-cell reactivity was measured by quantifying IFN-γ secretion in conditioned medium, presented as (mean ± SD) of 2–3 independent experiments. The results of the CRYAB analyses on the CSF-TCL for individual HLA-I alleles, of patients LP33 ($n = 2$) and LP51 ($n = 4$), are shown in multiple colors. The negative and positive controls were CSF-TCL incubated with Cos-7 cells, transfected with a control vector (vector) or untransfected Cos-7 cells (TCL), and the CSF-TCL were stimulated with P/I.

BLCL: blood-derived EBV-transformed B-cell lines; CRYAB: alpha B crystalline; CSF: cerebrospinal fluid; DAPI: 4', 6-diaminidino-2-phenylindole (DAPI); EBV: Epstein-Barr virus; mAb: monoclonal antibody; PCR: polymerase chain reaction; P/I: PMA and ionomycin; RNA: ribonucleic acid; TCL: T-cell lines.

restricted to the lytic EBV proteins, classified as immediate early (BRLF1), early (BaRF1 and BXLF1), and late (BCRF1 and BBRF3) proteins (Figure 3).

The antigen diversity and kinetic class of the EBV CD8 T-cell targets identified do not support CD8 T-cell cross-reactivity between EBV and neuroantigens, but agreed with the current concept that early lytic EBV proteins are prominent CD8 T-cell targets, respectively.[13,14] Herein, we provide the first evidence for CD8 T-cell recognition of the lytic EBV proteins BXLF1, BCRF1 and BBRF3. However, we did not confirm specific EBV proteins, especially EBNA1 and DNA polymerase (BALF5), as targets of the intrathecal EBV-specific T-cells in MS patients.[17,20,23] This discrepancy, likewise increased intrathecal EBV CD4 T-cell reactivity though indifferent between MS and OND patients as reported by two independent groups[19,22] is puzzling that needs follow-up. Despite differences on patient cohorts and experimental set-up, including autoBLCL versus peptides/lysates as antigens in functional assays on T-cells *ex vivo* versus CSF-TCL, the current data support the involvement of EBV-specific T-cells in the intrathecal inflammatory response in MS patients in the absence of a detectable intrathecal EBV infection.[17,19–23] Additional support for this notion is provided by recent studies on the experimental autoimmune encephalomyelitis (EAE) model in common marmosets, suggesting the pivotal role of CD20 B-cells, infected with the EBV-like simian γ-herpesvirus CalHV3, as the APC for the pathogenic T-cell response.[35]

Potential limitations of our study are: (1) a relatively large part of CSF-TCL from OND patients were generated several years ago, from a HuD-PNS study group (9/13 OND patients) and (2) the potential bias in data interpretation due to prior knowledge of the patients' diagnosis.[27] First, all CSF-TCL were generated and subsequently analyzed in functional T-cell assays in the same lab (Department of Viroscience, Erasmus Medical Center) according to the same in-house SOPs. Moreover, we showed that even long-term storage of CSF-TCL, particularly those generated from HuD-PNS patients, does not impair the immunocompetence of T-cells upon stimulation with P/I (Supplemental Figure 2). Second, the co-authors involved in generating and assaying the CSF-TCL for autoBLCL reactivity were blinded from the patients' diagnosis, except for the HuD-PNS patient cohort. Subsequent in-depth analyses (i.e. TCRBV, EBV target antigen and CRYAB T-cell analyses) of selected high autoBLCL responders were performed on CSF-TCL of unmasked CIS and MS patients. In our opinion, the use and interpretation of T-cell assays performed according to in-house SOPs by the same operators, using appropriate controls and settings, have contained this potential drawback of our study.

In conclusion, the current study provided novel insights into the association between EBV and MS. First, intrathecal T-cell reactivity towards autoBLCL was significantly increased in both CIS and MS patients, compared to other diseased controls. Second, in-depth analysis of four high autoBLCL T-cell responders demonstrated that the intrathecal autoBLCL-specific CD8 T-cell responses were oligoclonal and in two CSF-TCL directed toward the lytic EBV proteins, but not to CRYAB that was endogenously expressed by BLCL. The data suggested a local T-cell response to EBV in MS. This warrants further characterization of the magnitude and breadth of intrathecal EBV-specific T-cell responses using the whole EBV proteome in larger cohorts of patients and disease controls.

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Authors' contributions

GMGMV and RQH designed research; GPvN, JM and JGM performed research; JM contributed new reagents; GPVN, JGM and GMGMV analyzed data; and GPVN, JGM, JM, RQH and GMGMV wrote the paper.

Conflict of interest

The authors declare that there are no conflicts of interest.

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