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Cerebrospinal fluid CD4$^+$ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein

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Epstein-Barr virus–specific CD4$^+$ T cells could be involved in the pathogenesis of multiple sclerosis, provided they can gain entry to the intrathecal compartment. The authors have previously demonstrated that cerebrospinal fluid T cells from multiple sclerosis patients recognize autologous Epstein-Barr virus–transformed B cells. They now report that CD4$^+$ T cells specific for the Epstein-Barr virus DNA polymerase peptide EBV 627–641 were present in the cerebrospinal fluid from one of two multiple sclerosis patients, and that a high proportion of these CD4$^+$ T cells cross-recognized an immunodominant myelin basic protein peptide, MBP 85–99. In the observed patient, the proportion of EBV 627–641–specific CD4$^+$ T cells seemed to exceed 1/10 000 in cerebrospinal fluid, compared to approximately 1/100 000 in blood. These findings prove that Epstein-Barr–virus specific CD4$^+$ T cells can gain access to the intrathecal compartment, and suggest that Epstein-Barr virus–specific CD4$^+$ T cells could target myelin basic protein in the central nervous system. *Journal of NeuroVirology* (2004) **10,** 278–283.

Keywords: cerebrospinal fluid; Epstein-Barr virus; multiple sclerosis; myelin basic protein; specificity; T cells

Introduction

Several lines of evidence suggest that Epstein-Barr virus (EBV) could be one of the common environmental factors capable of inducing multiple sclerosis (MS) in genetically susceptible individuals (Ebers *et al*, 1995). Almost all MS patients have antibodies to EBV in their serum (Ascherio and Munch, 2000), and CD4$^+$ T cells recognizing autologous EBV-transformed B cells have been found in the cerebrospinal fluid (CSF) of MS patients (Holmøy and Vartdal, 2004). Furthermore, all the individuals in two clusters of MS showed evidence of infection with the same subtype of EBV (Munch *et al*, 1998). The risk of developing MS is positively correlated with the level of serum antibodies to EBV (Levin *et al*, 2003) and delayed primary EBV infection (Haahr *et al*, 1995). An association between EBV and MS etiology is compatible with population migration studies and the epidemic of MS seen in the Faroe Islands, both sets of evidence suggesting that MS is probably the result of some environmental insult occurring in early adolescence with a lengthy latency before symptom onset (Kurtzke, 2000).

MS is believed to be mediated by myelin-specific CD4$^+$ T cells. The immunodominant myelin basic protein (MBP) peptide MBP 85–99 is suggested as a likely target for pathogenic CD4$^+$ T cells (Ota *et al*, 1990). Activation of MBP-specific CD4$^+$ T-cells by molecular mimicry between MBP and viral antigens could thus explain the association between MS and EBV (Oldstone, 1987; Wucherpfennig and Strominger, 1995). In line with this hypothesis, a T-cell clone (TCC) from an MS patient has been demonstrated to cross-recognize MBP 85–99 and a peptide from EBV DNA polymerase (EBV 627–641), restricted

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by the HLA-DRB1*1501 and HLA-DRB5*0101 alleles on the DR2 haplotype (Lang *et al*, 2002). In order to be clinically relevant to the pathogenesis of MS, cross-reactive T cells must be able to gain access to the intrathecal compartment and should therefore be detectable within the CSF. To address this question, we looked for EBV 627–641–specific $CD4^+$ T-cells in the blood and CSF from two MS patients, and then tested these T cells for cross-recognition of MBP 85–99. Both patients studied were known to have a proliferative response to MBP in their blood. EBV 627–641–specific $CD4^+$ T cells were present in blood from both patients and in CSF from one patient. Eight out of 14 EBV 627–641–specific TCCs generated from CSF cross-recognized MBP 85–99. These results prove that EBV-specific $CD4^+$ T cells penetrate to the intrathecal compartment, where they could target an antigen in the myelin sheath.

Results

EBV 627–641–specific T cell lines (TCLs) were generated from blood in both patients, whereas EBV 627–641–specific CSF TCLs were only obtained from the first patient, MS1. The study was therefore focused on MS1. The CSF TCLs generated by repeated stimulation with the EBV 627–641 peptide (CSF TCL-2 and -3) responded to this peptide (Figure 1), whereas those generated by primary stimulation with interleukin (IL)-2 (CSF TCL-1) or initial stimulation with EBV 627–641 followed by IL-2 and phytohemagglutinine (PHA) (CSF TCL-4) did not. Only one of the two blood TCLs from MS1 generated by repeated stimulation with the EBV 627–641 peptide (blood TCL-1) responded to this peptide.

The precursor frequencies of EBV 627–641– and MBP 85–99–specific T cells in blood from MS1 were analyzed in a limiting dilution assay. The frequency of EBV 627–641–specific T cells was approximately 1/100 000 peripheral blood mononuclear cells (PBMCs), whereas the frequency of T cells responding to MBP 85–99 was approximately 1/50 000 PBMCs.

To determine whether the EBV 627–641–specific $CD4^+$ T cells from MS1 could cross-recognize an immunodominant MBP peptide, TCCs generated from CSF TCL-2, CSF TCL-3, and blood TCL-1 were simultaneously assayed against EBV 627–641 and MBP 85–99. Eight of 14 EBV 627–641–specific TCCs derived from CSF recognized MBP 85–99 (Figure 2). Only one EBV 627–641–specific TCC was derived from blood, and this clone did not recognize MBP 85–99 (data not shown).

To confirm that the EBV 627–641–specific $CD4^+$ T cells can be stimulated by EBV-infected B cells, we also tested whether these T cells responded to autologus EBV-transformed B cells (EBV-LCL). All EBV 627–641–specific TCLs and TCCs responded to autologous EBV-LCL, with stimulatory indexes from 6.6 to 49.3.

The human leukocyte antigen (HLA) restriction of the EBV-LCL and EBV 627–641–specific CSF TCLs are given in Figure 3. Two independent experiments demonstrated a predominant HLA-DP restriction in CSF TCL-3 (77% to 88% inhibition by anti-DP), whereas the proliferative response to autologous EBV-LCL in CSF TCL-2 was reduced by 70% by

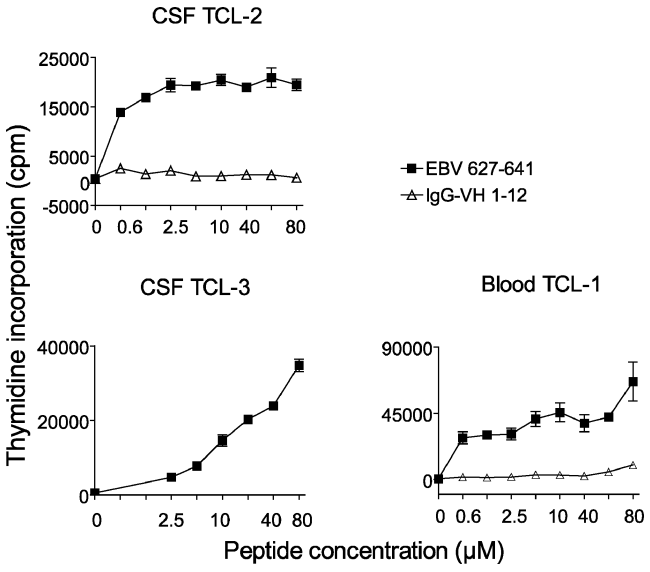

Figure 1 Proliferative responses of T-cell lines from cerebrospinal fluid (CSF TCL-2 and CSF TCL-3) and blood (blood TCL-1) from MS1 when stimulated with EBV 627–641 peptide and a control peptide from human IgG-VH. Due to limited amount of T cells, CSF TCL-3 was only tested against EBV 627–641, at concentrations of 2.5 to 80 μM. Values are mean cpm of triplicates, and error bars indicate standard deviation.

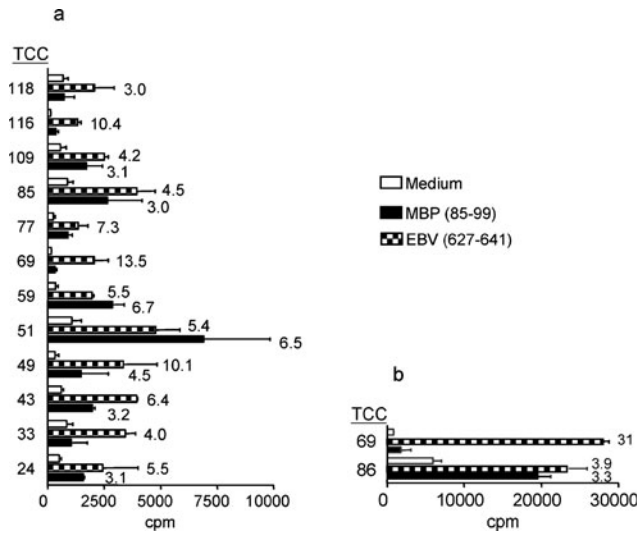

Figure 2 Proliferative responses of TCCs derived from CSF TCL-3 (**a**) and CSF TCL-2 (**b**) when stimulated with 30 μM MBP 85–99 or 30 μM EBV 627–641 peptide. Values are mean cpm of replicates, and error bars indicate standard error of mean. Stimulatory index of replicates with mean Δcpm exceeding 1000 are given at the end of each bar.

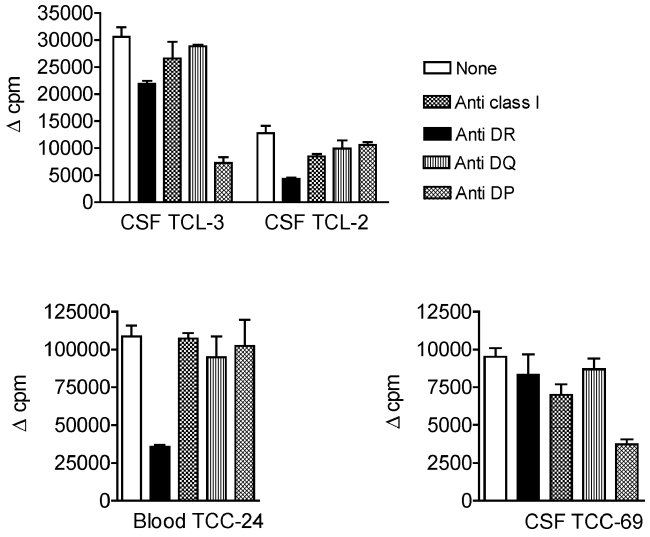

Figure 3 Proliferative T-cell responses to EBV in the presence and absence of monoclonal antibodies to HLA class I, HLA-DR, -DQ, and -DP molecules. CSF TCC-69 and blood TCC-24 were stimulated with EBV 627–641 presented by autologous PBMCs, and CSF TCL-2 and -3 were stimulated with autologous EBV LCL. Values are mean Δcpm of triplicates, and error bars indicate standard deviation. Mean and range cpm of unstimulated $CD4^+$ T cells and APCs (background) were CSF TCC-69: 589 (244–1378); PBL TCC-24: 1124 (250–2129); CSF TCL-2: 1049 (822–1440); CSF TCL-3: 797 (648–991).

anti-DR. Interestingly, EBV 627–641 induced proliferation of CSF TCC-69, which was also inhibited by anti-DP, whereas blood TCC-24 was restricted by HLA-DR.

In order to analyze the cytokine profile of EBV 627–641–responsive TCCs and TCLs from MS1, cytokine concentrations were measured in supernatants 48 h after stimulation of CSF TCC-24, CSF TCC-86, CSF TCL-1, CSF TCL-3, and blood TCL-1 with anti-CD3 (Table 1). CSF TCC-24 produced large amounts of both the Th1 cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α and the Th2 cytokines IL-5 and IL-4, compatible with a Th0 phenotype. On the other hand, CSF TCC-86 displayed predominant production of IFN-γ and TNF-α, indicating a Th1 phenotype. The TCLs produced much IFN-γ and no or little IL-4, suggesting a predominance of Th1 cells, although significant production of IL-10 and IL-5 shows that $CD4^+$ T cells producing Th2 cytokines were also represented in the EBV 627–641–responsive TCLs.

Discussion

This study is to our knowledge the first to demonstrate that $CD4^+$ T cells recognizing a defined EBV antigen and cross-recognizing MBP peptides occur in the intrathecal compartment. Hence, these data support a possible pathogenetic role for EBV-specific $CD4^+$ T cells in MS, although EBV has not been detected in MS brains (Morre *et al*, 2001). $CD4^+$ T cells recognizing both EBV and MBP peptides were present in both CSF TCLs from MS1 which had been generated by repeated stimulation with EBV 627–641, indicating that cross-reactive $CD4^+$ T cells are quite prevalent in the CSF of this patient.

The absence of proliferative responses in CSF TCL-1 and CSF TCL-4 from MS1 could reflect a lack of antigen-specific stimulation during cell culture, favoring expansion of IL-2–responsive cells with other specificities. Given that $CD4^+$ T cells constitute approximately 50% to 60% of the CSF cells (Cepok *et al,* 2001), each CSF TCL was derived from approximately 4000 $CD4^+$ T cells. As both CSF TCLs from MS1 generated by repeated peptide stimulation turned out to be EBV 627–641 responsive, the frequency of EBV 627–641–specific $CD4^+$ T cells in the CSF of this patient is probably in the same range as MBP-specific T cells in the spinal cord of rats with MBP-induced experimental allergic encephalomyelitis, which has been calculated to be 1/8000 (Ishigami *et al*, 1998). The precursor frequency of T cells specific for EBV 627–641 and MBP 85–99 in blood from MS1, as determined by limiting dilution, was considerably lower, consistent with the finding that only one of the blood derived TCLs from MS1 generated by repeated peptide stimulation of 200,000 PBMCs responded to EBV 627–641.

The observation that 8 of 14 EBV 627–641–specific CSF TCCs cross-recognized MBP 85–99 might seem surprising, given the high degree of specificity in the T-cell recognition of antigens. However, it is now

Table 1 Cytokline concentrations in supernatants from $CD4^+$ TCCs and TCLs 48 hours after stimulation with anti-CD3

T cells	*Stimulus*	*IFN-γ (pg/ml)*	*TNF-α (pg/ml)*	*IL-10 (pg/ml)*	*IL-5 (pg/ml)*	*IL-4 (pg/ml)*	*IL-2 (pg/ml)*
TCC-24	Medium	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
	Anti-CD3	28679.7	3062.3	166.4	1571.2	5365.6	<2.5
TCC-86	Medium	779.7	4.1	<2.5	<2.5	<2.5	<2.5
	Anti-CD3	26257.5	1117.6	266.9	499.4	36.1	<2.5
CSF TCL-1	Medium	<2.5	<2.5	<2.5	27.8	<2.5	<2.5
	Anti-CD3	8448.9	118.5	489.6	2671.0	<2.5	<2.5
PBL TCL-1	Medium	10.1	<2.5	<2.5	5.2	<2.5	<2.5
	Anti-CD3	24653.7	378.1	3883.0	2671.0	30.8	<2.5
CSF TCL-3	Medium	<2.5	>2.5	<2.5	13.0	<2.5	<2.5
	Anti-CD3	12719.7	99.1	1475.7	2560.0	<2.5	<2.5

hypothesized that cross-reactivity could be an essential feature of the T-cell repertoire, and that a single T cell must be able to recognize between 10^4 and 10^7 different nonamer peptides (Mason, 1998). In line with this unproven idea, it has been reported that more than 50% of MBP 93–105–specific TCCs from blood of MS patients could be activated by a synthetic peptide corresponding to residues 1 to 13 of human herpes virus 6 U24 (Tejada-Simon *et al,* 2003).

EBV 627–641–specific $CD4^+$ T cells displayed both HLA-DR and -DP restriction. The finding of DP restricted EBV 627–641–specific $CD4^+$ T cells was unexpected, given that EBV 627–641 has been reported to be presented to an MBP-specific TCC by the HLA-DRB5*0101-encoded molecule. Taken together, these observations suggest that the EBV 627–641 peptide is able to lodge into the peptide binding cleft of both HLA-DR– and -DP–encoded molecules. That a single peptide may bind to different HLA isotypes and elicit T-cell responses restricted by both HLA molecules have previously been demonstrated with other types of immunogenic peptides (Gedde-Dahl *et al*, 1992).

The observation that proliferating EBV 627–641–specific $CD4^+$ T cells produce huge amounts of IFN-γ could indicate that these cells are proinflammatory Th1 cells. However, these data must be interpreted with caution, as the repeated stimulation with antigen and IL-2 could have altered the cytokine profile. However, the stimulation procedure used during generation of the TCCs would be expected to select for memory T cells, which are less likely to change their cytokine profile during propagation *in vitro.* Interestingly, it was recently demonstrated that $CD4^+$ T cells in PBMCs from EBV-seropositive, but not EBV-seronegative, individuals produce IFN-γ upon stimulation with EBV. This could indicate that EBV-specific memory $CD4^+$ T cells are likely to be of a Th1 phenotype (Aymes *et al*, 2003).

EBV DNA polymerase is not constitutively expressed by EBV infected B cells *in vitro* (Thorley-Lawson, 2001), but is expressed during primary EBV infection (Furnari *et al*, 1992). Thus, EBV 627–641–specific memory $CD4^+$ T cell are most likely established during primary EBV infection. Delayed infection with EBV is associated with infectious mononucleosis and an increased risk of developing MS (Martyn *et al*, 1993; Haahr *et al,* 1995). The concept of heterologous immunity has been applied to explain the age dependent difference in the immune response to primary EBV infection (Selin *et al*, 1998; Thorley-Lawson, 2001). Younger individuals generally elicit a more powerful naive $CD8^+$ T-cell response that rapidly controls the infection. During aging, repeated infections add to the T-cell memory compartment so that the immune response against a delayed primary EBV infection could be more likely to recruit cross-reactive T cells.

The lack of EBV 627–641–specific $CD4^+$ T cells in CSF from MS2, in spite of proliferative responses to this peptide in $CD4^+$ TCLs from blood, could indicate that EBV 627–641–specific $CD4^+$ T cells were not present intrathecally in this patient. It is, however, not possible to draw a firm conclusion based on negative results. Given that the total number of cells in the original CSF sample from this patient was 21 000, the number of $CD4^+$ T cells obtained from CSF was approximately 10 000. The frequency of T cells specific for recall antigens like tetanus toxoid and purified protein derivative (PPD) is in the range of 100 to 500 per 10^6 T cells (Li Pira *et al*, 1999). Thus, the number of CSF cells may have been insufficient to ensure that EBV 627–641–specific $CD4^+$ T cells were included.

Activated T cells are able to cross the blood-brain barrier, and are retained intrathecally if they recognize their specific antigen within the CNS (Hickey *et al,* 1991). It is therefore possible that EBV-specific $CD4^+$ T cells activated in the periphery could penetrate into the CNS where those cross-reacting with a CNS antigen like MBP could be retained and reactivated intrathecally, where they proliferate and express their effector functions. This is in line with our finding of an increased frequency of EBV 627–641–specific $CD4^+$ T cells in CSF compared to blood in MS1, and the high proportion of cross-reactive TCCs that produce proinflammatory cytokines upon activation. In conclusion, these data support a pathogenetic role for EBV in MS by proving that EBV-specific $CD4^+$ T cells gain access to the intrathecal compartment, where they could be reactivated by MBP to proliferate and display effector functions like production of IFN-γ.

Materials and methods

Patients

MS1 is a 53-year-old female with relapsing remitting MS for 25 years, carrying HLA-DRB1*0801, HLA-DRB1*1501, HLA-DQB1*0402, and HLA-DQB1*0602. MS2 is a 38-year-old male suffering from secondary progressive MS, carrying HLA-DRB1*1501, HLA-DRB1*1501, HLA-DQB1*0602, and HLA-DQB1*0602. PBMCs from both patients had previously displayed brisk proliferative responses against MBP (Holmøy *et al*, 2003). The study was approved by the local ethical committee, and informed consent was obtained from the patients.

Generation of cell lines and clones

A sample of 25 ml CSF was collected by lumbar puncture. To avoid any contamination with blood, the first milliliters of CSF were discarded. The total number of CSF cells obtained from MS1 was 28 000, from MS2 21 000. The fraction of leukocytes in the CSF exceeded 95%, which makes it very unlikely that the CSF samples were contaminated with blood. The CSF cells were split into aliquots of approximately 7000 cells, which served as precursor cells for the CSF TCLs. Each TCL from peripheral blood

Table 2 Protocol for generation of $CD4^+$ T cell lines (TCLs) from blood and cerebrospinal fluid (CSF)

		Stimulation given at n days in culture			
Patient	*T-cell line*	*0*	*8*	*16*	*21*
MS1	Blood TCL-1	EBV 627–641	PHA	EBV 627–641	PHA
	Blood TCL-2	EBV 627–641	IL-2	EBV 627–641	PHA
	CSF TCL-1	IL-2	PHA	PHA	PHA
	CSF TCL-2	EBV 627–641	PHA	EBV 627–641	PHA
	CSF TCL-3	EBV 627–641	IL-2	EBV 627–641	PHA
	CSF TCL-4	EBV 627–641	IL-2	PHA	PHA
MS2	Blood TCL-1	EBV 627–641	PHA	EBV 627–641	PHA
	Blood TCL-2	EBV 627–641	IL-2	EBV 627–641	PHA
	CSF TCL-1	EBV 627–641	PHA	EBV 627–641	PHA
	CSF TCL-2, 3	EBV 627–641	IL-2	EBV 627–641	PHA

was derived from 200000 PBMCs. The stimulation procedure for each TCL is given in Table 2. The cells were maintained in RPMI 1640 (Gibco, Paisley, Scotland), supplemented with 10% autologous (first week of culture) or pooled human serum. Irradiated (25 Gy) autologous PBMCs served as antigen presenting cells (APCs). PHA (Murex, Dartford, UK) was used at 3 μg/ml. IL-2 (Amersham, Buckinghamshire, UK) was used at 10 international units/ml. Peptides were purchased from Neosystem (Strasbourg, France), and used at a concentration of 30 μM in all stimulations during TCL generation, because this concentration has been shown to be adequate (Lang *et al*, 2002). A peptide from human immunoglobulin G variable heavy region, with amino acid sequence QFQLVESGGGL, was used as a control. EBV 627–641–specific TCLs from MS1 were cloned at 0.3 or 1 cell per well in the presence of 20 000 irradiated heterologous PBMCs, IL-2, and PHA. The cloning frequency was 6% to 23%, which makes it highly likely that the clones are genuinely monoclonal (Fitch and Gajewski 1993). The clones were expanded with allogeneic feeder cells, PHA, and IL-2 after 8 days. The phenotype of the TCLs and TCCs was tested in a rosetting assay with immunomagnetic beads coated with anti-CD4 and anti-CD8 (Dynal ASA, Oslo, Norway) (Gaudernack and Lundin, 1989). The proportion of $CD4^+$ cells exceeded 95%.

EBV-LCLs were generated from peripheral blood by incubating 10×10^6 PBMCs with a supernatant from a B95-8 EBV-infected marmoset cell line.

Proliferation assays

The specificity of the TCLs and TCCs was tested by incubation of 1.0×10^5 $CD4^+$ T cells with 1.0×10^5 APCs, which had been preincubated with antigen overnight. Cells were cultured for 72 h, and 1 μCi of [methyl-^{3}H]-thymidine was added for the last 16 h. Proliferation was measured as counts per minute (cpm). The wells were considered to be positive when the ratio between stimulated and unstimulated cells (the stimulatory index, SI) exceeded 3, and Δcpm (cpm of antigen stimulated cells after subtraction of cpm of unstimulated cells) exceeded 1000 (Zhang *et al*, 1994).

Limiting dilution assay (LDA)

The proportion of EBV 627–641–specific blood-derived T cells also recognizing MBP 85–99 was established using LDA. LDA was performed by seeding replicates of 24 wells with 100 000 to 3000 PBMCs in twofold dilutions with 50 000 irradiated PBMCs (25 Gy), which had been preincubated with 30 μM peptide. Thymidine incorporation was measured after 7 days. Wells were scored positive if the cpm of stimulated wells exceeded twice the mean cpm of unstimulated wells, and exceeded the mean cpm of unstimulated wells + 3 SD (Pender *et al*, 2000). The cell number yielding 37% negative wells gives a crude estimate of the reciprocal frequency of antigen-specific cells (Fazekas de St Groth, 1982).

HLA blocking experiments

The HLA restriction of the CSF-derived $CD4^+$ T cells was analyzed by incubating EBV 627–641–responsive CSF TCLs with irradiated (80 Gy) autologous EBV-LCLs in the presence and absence of 20 μg/ml monoclonal antibodies to HLA class I (cloneW6/32; IgG2a; American Tissue Culture Collection [ATCC], Manassas, VA, USA), HLA-DR (clone L243; IgG2a; ATCC), HLA-DQ (clone SPVL-3; IgG2a; gift from Dr. Hergen Spits, Netherlands Cancer Institute, Amsterdam, The Netherlands), HLA-DP (clone B7/21; IgG3; gift from I. Trowbridge, The Salk Institute, La Jolla, CA, USA), and a monoclonal IgG2a and IgG3 isotype control (Diatec, Oslo, Norway).

Cytokine assays

The cytokine profile of proliferating $CD4^+$ T cells was determined by incubating 100 000 $CD4^+$ T cells in 200 μl medium in 96-well flat bottom plates that had been coated with either 3 μg/ml anti-CD3 (Diatec, Oslo, Norway) or phosphate-buffered saline (PBS). Concentrations of TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-10 were measured in supernatants harvested 48 h after stimulation, by using a Th1/Th2 Human Cytokine BD Cytometric Bead Array Kit (Becton, Dickinson & Co, Franklin Lakes, NJ, USA) as described by the manufacturer.

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