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Cross-reactivity of autoreactive T cells with MBP and viral antigens in patients with MS

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1. ABSTRACT

In this study, we detected the viral DNA of Human Herpes Virus 6 (HHV-6) in the sera and cell-free cerebrospinal fluid (CSF) of Chinese multiple sclerosis (MS) patients. The results revealed that the copy numbers of serum HHV-6 viral DNA were higher in MS than in normal subjects (NS) or in other neurologic diseases (OND). We also found that in the MS subjects, most T cells recognizing myelin basic protein (MBP) were cross-reactive and could be activated by a synthetic peptide corresponding to residues of HHV-6 or EBV. The estimated precursor frequency of these cross-reactive T cells recognizing both peptides, MBP and HHV-6 or EBV, was significantly elevated in MS compared with that in controls. More significant was the presence of CD8+ cytotoxic cross-reactive T cells, as they could directly induce injury to oligodendrocytes that are known to express both MBP and MHC class I molecules. The study provides important evidence for understanding the potential role of HHV-6 or EBV infection in the pathogenesis of MS.

2. INTRODUCTION

Multiple sclerosis (MS) is believed to be an autoimmune disease in which the body's own defense mechanisms (immune system) seem to mistakenly attack and destroy myelin, the insulating material surrounding the nerve fibers. The destruction of myelin is directly related to the neurological problems experienced by MS patients, including weakness and loss of sensation (1,2). The mechanism of myelin destruction is still not understood, but is thought to involve dysfunction of the body's own immune system in response to self myelin protein components (3-7). It has been speculated that in MS patients the immune system produces abnormal cells capable of attacking myelin. These abnormal cells can be found in the brains of MS patients and have been proven to cause an MS-like disease in animal models (3,8). Another important feature about MS is that the disease is associated with certain viral infections. Increased activity of certain viruses has been found in the blood and brains of MS patients. In this study, we detected HHV-6 viral DNA in

Table 1. Peptide sequences

Name	Peptide sequences	For inducing cell
HHV-6 $(U24)_{1-13}$	RPRTPPPSY	CD8+
MBP_{83-89}	TPRTPPPQ	
$EBV_{627-642}$	TGGVYHFVKKHVHES	CD4+
MBP_{81-95}	ENPVVHFFKNIVTPR	

serum and cell-free CSF using a nested RT-PCR technique. We also detected an association between anti-HHV-6 / EBV antibody and myelin basic protein (MBP) in these MS patients. One theory is that some viruses may be able to activate abnormal myelin-attacking cells in MS because they have structural similarities with myelin (9-11). Therefore, cells produced by the immune system to attack virus can also recognize and attack the myelin. This theory is particularly interesting and relevant to MS, because it may explain the mechanism for suspected association of viral infections with MS (11). Research in the area of viral infection has indicated that two important viruses, human herpes virus-6 (HHV-6) and Epstein-Barr virus (EBV), are likely to activate myelin-attacking cells, because they have structural similarities with myelin (12-15). In this study we investigated the role of HHV-6 and EBV in the activation of myelin-attacking cells in Chinese MS patients, and the mechanism associated with this activation process. Peptides have been made to match the sequences of HHV-6 and EBV that have similarities with myelin (11). These peptide sequences have been tested on blood of Chinese MS patients to determine whether they can activate abnormal myelin-attacking cells. The experiments also addressed the important question as to whether these abnormal myelin-attacking cells found in Chinese MS patients are indeed activated by increased activity of HHV-6 and EBV and the results showed that they have the ability to directly induce myelin destruction (16). The results of this paper provide important information for the understanding of how abnormal myelin-attacking cells are activated in MS and for determining the role of HHV-6 and EBV in the disease processes of Chinese MS patients.

3. MATERIALS AND METHODS

3.1. Patients and specimens

Twelve patients with relapsing-remitting MS or secondary progressive Chinese MS patients, as determined by the Poser criteria, were included. Patients had not been treated with immunosuppressive or immunomodulatory drugs for at least 3 months before entering the study. The study protocol was approved by the Medical Ethics Review Board of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai Ruijin Hospital and Shanghai Xinhua Hospital. PBMCs were prepared from heparinized blood specimens by Ficoll-Hypaque gradient separation. Serum and CSF were collected from patients of MS by centrifugation.

3.2. Reagents and peptides

Peptide sequences are previously published (17). Reagents and peptides are shown in Tables 1 and 2.

3.3. HLA genotyping

Total cellular RNA was extracted from PBMC specimens using the RNeasy Mini Kit. RNA was reverse transcribed to first-strand cDNA using an Oligo-dT primer. cDNA was amplified by PCR using oligonucleotide primers specific for DR/DQ. Briefly, 1 µl cDNA was added to the following amplification mixture: 5 µl 10×PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3 µl 25 mM magnesium chloride, 1 µl 10 mM dNTP mix, 0.3 µl Taq polymerase (5 U/µl) (AmpliTaq Gold, Perkin Elmer, Norwalk, CT, USA), 10 pmol of each specific forward and reverse primer. The amplification profile used was 1 min at 95^{o}C for denaturation, 20 sec at 65^{o}C for annealing, 40 sec at 72^{o}C for extension for a total of 30 cycles. The amplified PCR products were separated in 1% agarose gels by electrophoresis and stained with ethidium bromide for visualization.

3.4. Detection of HHV-6 DNA in serum and cell-free CSF

Viral HHV-6 DNA was detected in cell-free serum specimens from subjects using nested PCR and Southern hybridization with specific primers and probes. The results are expressed as detectable (+) (> 5 DNA copies/µl) (11,16). Briefly, cell-free DNA was extracted from serum specimens using the QIAmp DNA purification method (Qiagen) according to the manufacturer's instructions. To achieve the highest sensitivity for detection of viral DNA, the extracted DNA was amplified by nested PCR followed by Southern hybridization. The outer primer is a 101K ORF primer, the inner primers are a sense primer for the F2 site and an antisense primer for the F3 site (9,18). The sensitivity of the nested PCR has been estimated using quantitative PCR amplification and hybridization using a serial dilution of pH6Z-3001, which allowed the detection of five copies of pH6Z-3001. For all experiments, 5 µl of extracted DNA was subjected to a first-stage PCR in a total reaction volume of 20 µl for 35 cycles. The second-stage PCR was performed by adding the PCR products from the first-stage PCR to a second reaction volume for 30 cycles. The second-stage PCR products were subjected to 1% agarose gel electrophoresis and then vacuum-blotted to positively charged nylon membrane. The F2 antisense primer was labeled with (^{32}P) ATP at the 5' terminus using T4 polynucleotide kinase (Biolabs) to use as the probe for Southern hybridization. All serum specimens were evaluated within one set of experiments to minimize variation between individual specimens.

3.5. Determination of BV gene usage in CSF and in blood T cells by real-time PCR analysis

Quantitative real-time RT-PCR was performed on an ABI Prism 7000 sequence detection system. BC was used as a reference for sample normalization (19). Total RNA isolated from PBMCs or T cells was reverse-transcribed into cDNA using random hexamer. Relative quantification of gene expression was calculated using the delta CT method based on signal intensity of the PCR reactions according to the following formula: TCR BVn (%) = (2– (BVn Ct – BC Ct) / Σ (2– (BV1–25 Ct – BC Ct)))×100 TCR V gene. Briefly, total RNA was extracted from isolated T cells using an RNeasy kit (Qiagen). First-strand cDNA was reverse transcribed from total RNA and subjected to PCR amplification with a set of 25 primers

Table 2. TCRBV sequences

Name	Sequence	
	5' to 3'	3'to 5'
BV1	AAGCACCTGATCACAGCAACT	TAGTTCAGAGTGCAAGTCAGG
BV2	GGTTATCTGTAAGAGTGGAACCT	AGGATGGGCACTGGTCACTGT
BV3	TCGAGATATCTAGTCAAAAGGACG	GGTGCTGGCGGACTCCAGAAT
BV4	AAGCAGGGATATCTGTCAACGT	TTCAGGGCTCATGTTGCTCAC
BV5	GATCAAAACGAGAGGACAGCA	AGCACCAAGGCGCTCACATTCA
BV6	CTCAGGTGTGATCCAATTTCA	CCCCCGCTCTGTGCGCTGGAT
BV7	CATGGGAATGACAAATAAGAAGTCT	TGGCTGCAGGGCGTGTAGGTG
BV8	CCCCGCCATGAGGTGACAGAG	GAGTCCCTGGGTTCTGAGGGC
BV9	CCAAAATACCTGGTCACACAG	CCAGGGAATTGATGTGAAGATT
BV10	ACCTAGACTTCTGGTCAAAGCA	GGACTGGATCTCCAAGGTACA
BV11	TTATAGGGACAGGAAAGAAGATC	ATGTGAGGGCCTGGCAGACTC
BV12	CAAGACACAAGATCACAGAGACA	GGCAGCAGACTCCAGAGTGAG
BV13	TGAAGACAGGACAGAGCATGACA	CACAGATGTCTGGGAGGGAGC
BV14	ACCCAAGATACCTCATCACAGTG	AGAGGTCTGGTTGGGGCTGGG
BV15	TCACAAAGACAGGAAAGAGGATT	GGGGATGGCAGACTCTAGGGA
BV16	GTTCCCCAGCCACAGCGTAATA	CAGTTCTGCAGGCTGCACCTT
BV17	GTCCCCAAAGTACCTGTTCAGA	AGCTGTCGGGTTCTTTTGGGC
BV18	AGACACCTGGTCAGGAGGAGG	TGCCGAATCTCCTCGCACTAC
BV19	CCAGGACATTTGGTCAAAGGAAAA	CAGTGCCGTGTCTCCCGGTTC
BV20	GACCCTGGTGCAGCCTGTG	GAGGAGGAGCTTCTTAGAACT
BV21	CCCAGATATAAGATTACAGAGAAA	CTGGATCTTGAGAGTGGAGTC
BV22	CACAGATGGGACAGGAAGTGATC	GTCCTCCAGCTTTGTGGACCG
BV23	AAGAGGGAAACAGCCACTCTG	CAGCTCCAAGGAGCTCATGTT
BV24	CCAAGATACCAGGTTACCCAGTTT	CAGGCCTGGTGAGCGGATGTC
BV25	AAAACATCTTGTCAGAGGGGAA	TGAATCCTCAAGCTTCGTAGC
TCRBC	CAGCGCCCTTGTGTTGATG	AAGCGCTGGCAAAAGAAGAA

specific for TCR BV genes whose sequences have been previously published (19). The amplified PCR products were separated in a 1% agarose gel by electrophoresis and then transferred to positively charged nylon membrane using a vacuum blotter at 5 mHg for 90 min. DNA was fixed to the membrane by a 3 min exposure to UV crosslinking and prehybridized at 68^{o}C for at least 1 hour. 0.1 mg/ml of poly (A) was then added to the hybridization solution (5×SSC, 1% blocking solution, 0.1% N-lauroylsarkosine, 0.02% SDS) to reduce non-specific binding to non-target DNA. Hybridization temperature and washing conditions were optimized for different probes to ensure stringent hybridization. Probes were labeled with digoxigenin-dUTP and terminal transferase (Boehringer Mannheim). Hybridization was performed for 6 hr in a buffer containing 5×SSC, 1% blocking solution, 0.1% N-lauroylsarkosine, 0.02% SDS. Detection of hybridized DNA was performed with an anti-digoxigenin antibody and a chemiluminesencent substrate, disodium 3- (4-methoxyspiro{1,2-dioxetane-3,2'- (5'-chloro) tricyclo (3.3.1.1) decan}-4-yl) phenyl phosphate (Boehringer Mannheim). Membranes were exposed to X-ray film for 15-30 min at room temperature. PCR products were quantified using a Gel Doc 1000 scanning densitometer (Bio-Rad). Purified PCR products were directly sequenced using the T7 sequencing kit. 1.5 µg of template was sequenced with 2 pmol of the corresponding V gene primer using the dideoxy chain termination method.

3.6. Quantitative measurement of MBP

Quantitative measurement of MBP in CSF or serum of MS Samples was performed using the DSL-10-58200 ACTIVE® MBP ELISA Kit.

3.7. The detection of HHV-6 IgG antibody

HHV-6 IgG antibody in human serum or plasma was measured using an ELISA for Human Herpesvirus-6 (HHV-6) IgG antibody (Advanced Biotechnologies).

3.8. Epstein-barr virus VCA IgM (mu)-capture ELISA

An enzyme immunoassay for the qualitative and quantitative determination of IgM antibodies against the viral capsid antigen (VCA) of Epstein-Barr Virus (EBV) in human serum and plasma was used.

3.9. Epstein-barr virus EBNA-1/ VCA IgG ELISA

An enzyme immunoassay for the qualitative and quantitative determination of IgG antibodies against the Epstein-Barr Virus nuclear antigen (EBNA-1) in human serum and plasma was used.

3.10. Precursor frequency analysis of $CD4^+$/$CD8^+$ T cells recognizing MBP and HHV-6 peptides

PBMCs were seeded at 3×10^5 cells per well in 96-well U-bottomed plates in the presence of the MBP_{83-89} or HHV-6 $(U24)_{1-13}$ peptides at 30 µg/ml. Medium alone was used as a control. A total of 24 wells were plated for each antigen. Seven days later, the cultures were tested for reactivity to both peptides. Each culture was split into three identical aliquots and tested in duplicate for reactivity to MBP and the indicated peptides. Two aliquots were tested for reactivity to peptide MBP_{83-89} and peptide HHV-6 $(U24)_{1-13}$, both at 30 µg/ml, in the presence of irradiated (6,000 rads) autologous PBMCs (10^5 cells per well) as a source of APC. One aliquot served as a control (medium alone in the presence of APC). The reactivity of the end cultures was measured after 72 hours by proliferation assays as described elsewhere in this study. A T cell line was defined as reactive to a given peptide when the CPM was greater than 1,000 and exceeded the CPM of the control (medium alone) and of the other peptide by at least three-fold. A cross-reactive T cell line was defined when the CPM of both peptides exceeded 1,000 and the control CPM by at least three-fold. The frequency of peptide-

reactive T cells was then estimated by dividing the number of positive wells by the total number of PBMCs seeded in the initial culture (20).

3.11. Estimation of the frequency of specific T cells

PBMCs were first processed to deplete $CD4^+/CD8^+$ T cells using magnetic Dynal beads (cell to bead ratio: 1:10). The resulting $CD4^+/CD8^+$ T cell-depleted PBMCs were then plated out at 200,000 cells /well in the presence of the HHV-6/EBV peptide (20 μg/ml). The cell number per well was previously pre-determined as an optimal cell density to detect specific T cells in this system. Seven days later, each culture was examined for specific proliferation to the HHV-6 /EBV peptide in proliferation assays. Briefly, each well was split into four aliquots (approximately 10^4 cells per aliquot) and cultured in duplicate with irradiated (6000 rads) autologous PBMCs (10^5 cells per well) in the presence and absence of the peptide. Cultures were kept for three days and pulsed with (^{3}H)-methylthymidine at 1 μCi per well during the last 16 hours of culture. Cells were then harvested using an automated cell harvester and (^{3}H)-methylthymidine incorporation was measured in a Microbeta Trilux counter. A well/culture was defined as specific for the corresponding peptide when the CPM was greater than 1,500 and exceeded the reference CPM (in the absence of the antigen) by at least three-fold. The frequency of specific T cells was then estimated by dividing the number of specific wells by the total number of $CD4^+/CD8^+$ T cell predepleted PBMCs seeded in the initial culture (21).

3.12. The reactivity pattern of resulting T cell lines

The resulting T cell lines were examined for their reactivity to MBP_{83-89} and HHV-6 $(U24)_{1-13}$ peptides or to a control peptide (T cell receptor sequence: ASSENRASYNEQFFG) at a concentration of 30 μg/ml in the presence of irradiated autologous antigen-presenting cells. Cell proliferation was expressed as stimulation index (experimental CPM / CPM in medium alone) ± standard deviation. Cross-reactive T cell lines are indicated as: reactivity to the peptide used in primary stimulation / reactivity to the other peptide. 10,000 to 20,000 cells of each T cell line were cultured with irradiated autologous PBMCs (100,000 cells per well) in the presence of the indicated peptides (30 μg/ml). Duplicate wells were prepared for each peptide per T cell line.

3.13. Cytotoxic activity was measured in a standard ^{51}Cr-release assay

An aliquot of each $CD8^+$ T cell line was cultured with irradiated autologous PBMCs in the presence and absence of the HHV-6/EBV peptide or the MBP peptide for 3 days and pulsed subsequently with (^{3}H)-thymidine at 1 μCi per well during the final 16 hour of culture. Cells were harvested and incorporation was measured in a beta-plate counter. $CD8^+$ T cell lines were examined for cytotoxicity towards autologous EBV-transformed B cells that were pulsed with the corresponding peptides (HHV-6/EBV cells and MBP cells) and labeled with ^{51}Cr at an effector to target cell ratio of 10. Unpulsed autologous EBV-transformed B cells were used as control target cells. Cytotoxic activity was measured in a standard ^{51}Cr-release assay. The maximum and spontaneous release was determined in wells containing detergent or medium alone. The percent specific cytolysis was calculated as (experimental release – spontaneous release / maximum release – spontaneous release) × 100%. In parallel, cross-reactive T cell lines were tested for specific cytotoxicity against autologous target cells pulsed with the MBP peptide in the presence and absence of a monoclonal antibody to HLA class I used at the concentration of 20 μg/ml (4,18).

3.14. The phenotypic expression of the representative CD8+ cross-reactive T cell lines

$CD8^+$ T cell lines were examined for the expression of CD4/CD8, CD45RA/CD45RO and TCR (alpha) (beta)/TCR (gamma) (delta) using monoclonal antibodies conjugated with FITC or PE by direct dual staining and flow cytometric analysis. Briefly, 1×10^5 cells of each T cell line were washed in PBS containing 1% FBS and 0.1% sodium azide (FBS-PBS) and resuspended in 100 μl FBS-PBS containing fluorochrome-labeled antibodies (CD4/CD8, CD45RA/CD45RO, TCR (alpha) (beta)/TCR (gamma) (delta), Becton Dickinson) or appropriate Ig isotype controls. After incubation for 30 min, the cells were washed three times in FBS-PBS, and analyzed using a FACS Calibur.

4. RESULTS

4.1. Characteristics of cell-free serum/CSF HHV-6 DNA and HLA-DR genotypes in Chinese MS patients

A group of 12 patients with relapsing-remitting or secondary progressive MS was included in the study. First, we detected cell-free serum and CSF HHV-6 viral DNA in all subjects using nested RT-PCR (Tables 3 and 4). The results revealed that the copy numbers of HHV-6 viral DNA in serum and cell-free CSF were higher in MS patients than in NS or OND patients (Figure 1).

4.2. The association between anti-HHV-6/EBV antibody and MBP in Chinese MS Patients

There was a high titer of serum anti-HHV-6 antibody in 12 MS patients (OD Ratio=2.45±1.58). Furthermore, it was also found that anti-HHV-6 antibody was neither in the CSF of MS patients (OD Ratio=0.087±0.105) nor in the serum of the controls (OD Ratio=0.018±0.021). Similarly, the increase in the titer of serum antibodies to EBNA-1 (OD=2.07±0.82 or 1865.08±1721.65 U/ml) and VCA (OD= 1.84±0.51 or 1631.10±1134.13 U/ml) was significant in the MS patients. In contrast, CSF antibodies to EBNA-1 and VCA (CSF: EBNA-1 OD=0.096±0.027 or 2.78±3.11 U/ml and VCA: OD=0.13±0.03 or 13.12±3.13 U/ml) were less than those in the sera of MS patients. Interestingly, a higher concentration of MBP antigen was detected in CSF than that in serum of MS patients (CSF: OD=1.53±1.38 or 7.44±9.99 ng/ml; Serum: OD=0.15±0.03 or 0.04±0.02 ng/ml, $P<0.001$; Table 5).

4.3. Cross-reactivity of $CD4^+/CD8^+$ autoreactive T cells between HHV-6 and MBP

This study was undertaken to examine the potential role of HHV-6/EBV in the activation of MBP-

Table 3. Clinical characteristics, presence of cell-free serum/CSF HHV-6 DNA and HLA genotypes of Chinese MS patients

Subjects	Sex	Age	Type of Disease	Cell free serum/CSF HHV-6 DNA	HLA-DR genotypes MS		
					DRB1* (M)	DRB1* (H)	DRQB1*
MS4	M	55	RR	- /-	DRB1*02/14	1502	/
MS5	F	63	RR	+/+	DRB1*04/11	0401	DQB1*07/08
MS7	F	43	RR	+/+	DRB1*04/09	0406	DQB1*08/09
MS8	F	33	RR	+/-	DRB1*02/07	1501	DQB1*02/06
MS9	F	61	SP	+/+	DRB1*03/14	0301	/
MS10	F	39	SP	+/+	DRB1*04/13	0405	DQB1*04/06
MS11	F	47	RR	+/+	DRB1*02/16	1501 1601	DQB1*05/06
MS12	F	45	RR	+/-	DRB1*03/09	0301	DQB1*03
MS13	F	39	RR	+/+	DRB1*04/08	0406	DQB1*03/05
MS14	F	59	RR	-/-	DRB1*04/13	0403	DQB1*03/05
MS15	M	47	SP	+/-	DRB1*12/08	/	DQB1*03/05
MS16	M	48	SP	-/+	DRB1*01/01	/	/

Abbreviations: RR, relapsing-remitting MS; SP, secondary progressive MS. Cell-free viral DNA for HHV-6 was detected in serum specimens derived from the subjects using nested PCR and Southern hybridization with specific primers and probes. The results are expressed as detectable (+) (> 5 DNA copies/μl) and undetectable (-).

Table 4. HLA-DRB1* (DR4) allele represents the most dominant genotype in Chinese MS patients

	DRB1*02		DRB1*03		DRB1*04	
Normal Control	42	0.140	15	0.050	28	0.093
MS patients	3	0.250	2	0.167	5	0.417

Table 5. Association of Anti-HHV-6/EBV Antibody and MBP in Chinese MS Patients

Subject	Sex	Age	MBP (Ag)		ANTI-HHV-6 (IgG)		ANTI-EBV-VCA (IgG)		ANTI-EBV-EBNA-1 (IgG)		ANTI-EBV-VCA (IgM)	
			CSF	SERUM	CSF	SERUM	CSF	SERUM	CSF	SERUM	CSF	SERUM
MS4	M	55	+	-	-	+	±	+	-	+	-	-
MS5	F	63	-	-	-	+	-	+	-	+	-	-
MS6	F	22	+	-	-	+	-	+	-	+	-	-
MS7	F	43	+	-	-	+	-	+	-	+	-	-
MS8	F	33	+	-	-	+	-	+	-	+	-	-
MS9	F	61	N	-	N	+	N	+	N	+	N	-
MS10	F	39	+	-	N	±	N	+	N	+	N	-
MS11	F	47	N	-	N	±	N	+	N	+	N	-
MS12	F	45	N	-	N	+	N	+	N	+	N	-
MS13	F	39	+	-	-	+	-	+	-	+	-	-
MS14	F	59	+	-	-	+	±	+	-	+	-	-
MS15	M	47	+	-	-	+	-	+	-	+	-	-
MS16	M	48	N	-	-	-	N	+	N	+	-	-

An ELISA Kit was used for the detection of anti-HHV-6 IgG antibody/EBV IgG/IgM antibody and a highly specific two-site ELISA Kit was used for the detection of MBP antigen in the CSF and serum of Chinese MS patients.

reactive T cells through T cell recognition of the shared sequence region between HHV-6 and MBP (for induction of $CD8^+$ T cells, HHV-6 peptide: RPRTPPPSY / MBP_{83-89} peptide: TPRTPPPQ and for induction of $CD4^+$ T cells, $EBV_{627-642}$ peptide: TGGVYHFVKKHVHES / MBP peptide: ENPVVHFFKNIVTPR; Figure 2). We examined the T cell responses to two synthetic peptides containing an identical core sequence by estimating the precursor frequency of the T cells in PBMCs. Cultures were primed with peptide MBP_{83-89} or peptide HHV-6 $(U24)_{1-13}$ and tested 7 days later for specific reactivity to the two peptides in reference to a medium control. As shown in Figure 2, The estimated frequency of T cells specific for the two peptides was significantly higher in Chinese MS patients than in controls (0.60×10^{-6} vs. 0.20×10^{-6} for MBP_{83-89} peptide and 0.53×10^{-6} vs. 0.20×10^{-6} for HHV-6 peptide ($p<0.01$). The estimated frequency of the cross-reactive T cells primed by peptide MBP+HHV-6 $(U24)_{1-13}$ was 1.20×10^{-6} in Chinese MS patients. A high precursor frequency of $CD4^+$ T cells reactive to both peptides was also observed in controls (0.68×10^{-6}, 0.65×10^{-6} and 1.10×10^{-6}). Similarly, cross-reactive T cells were also detected in cultures primed with peptide MBP_{83-89} + MBP_{81-95} or HHV-6 $(U24)_{1-13}$ + $EBV_{627-642}$ in Chinese MS patients as well as in single MBP, HHV-6 or EBV peptide controls (1.25×10^{-6},1.12×10^{-6} vs. 0.60×10^{-6}, 0.53×10^{-6}, or 0.68×10^{-6}, 0.65×10^{-6} $p<0.05$). These results suggest that a significant fraction of T cells recognizing the 83-89 and 81-95 regions of MBP could be fully activated to proliferate by HHV-6 $(U24)_{1-13}$ or $EBV_{627-642}$ peptides that share sequence homology with the MBP peptide.

4.4. The reactivity pattern and TCR V gene usage of cross-reactive T cells

To confirm that the T cells recognizing both HHV-6 $(U24)_{1-13}$ and MBP_{83-89} peptides result from the same T cell clone, we selectively generated T cell lines and subsequently characterized their reactivity pattern to the peptides. The resulting 12 T cell lines could be categorized, according to their reactivity to the peptides, into specific T cell lines and cross-reactive T cell lines. The reactivity of each T cell line was consistent with that of the parental T cell line. The resulting cross-reactive T cell lines examined

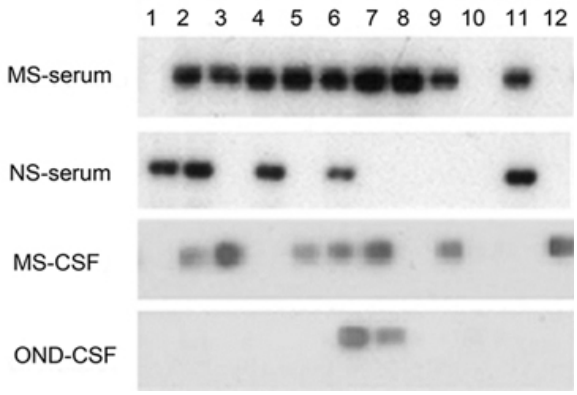

Figure 1. Cell-free serum and CSF DNA for HHV-6. The Figure revealed that the incidence of serum (9/12, 75.00%) and cell-free CSF (7/12, 58.33%) HHV-6 viral DNA was higher in the MS group than in the NS (5/12, 41.67%) or OND (2/12, 16.67%) groups.

were found to preferentially use TCR BV_{13} (Table 6 and Figure 4).

4.5. Cross-reactivity of $CD8^+$ cytotoxic T cells between HHV-6 and MBP

Based on the findings described above, we hypothesized that the core sequence/epitope shared between MBP and HHV-6 antigen may sensitize a subset of $CD8^+$ cytotoxic T cells that are able to cross-react with both antigens during the course of HHV-6 re-activation in Chinese MS patients. These $CD8^+$ cytotoxic cross-reactive T cells may have significant pathological relevance in MS as they could directly injure oligodendrocytes that are known to express both MBP and MHC class I molecules (HLA-A2, A11). It has been demonstrated that $CD8^+$ cytotoxic MBP-reactive T cells are able to cause CNS inflammation and extensive demyelination in EAE, which closely resembles CNS lesions in the MS brain (Table 7 and Figure 4).

4.6. Phenotypes of the representative cross-reactive CD8+T cell lines

The cytotoxic activity of $CD8^+$ cross-reactive T cell lines derived from $A2^+$or $A11^+$ Chinese MS patients was also analyzed. It was found that an increased proportion of $CD8^+$ T cells primed by HHV-6 U24 peptide are cross-reactive with MBP and are cytotoxic toward target cells pulsed with the peptides in the context of HLA class I. The obtained $CD8^+$ T cell lines expressed exclusively CD8 and (alpha) (beta) TCR, and belonged to the $CD45RA^-/CD45RO^+$ memory T cell subset (Figure 5).

5. DISCUSSION

In this study, the molecular mimicry mechanism and the distinctive mechanisms of $CD4^+$ and $CD8^+$ cross-reactive T cells have been investigated in Chinese MS patients. It has been speculated, based on an increasing body of evidence, that myelin-reactive T cells may be activated by microbial agents, in particular, viral antigens during the course of infection and re-activation through a mechanism known as molecular mimicry (11,22,23). In a potential association with a functional deficit in immune regulation, these autoreactive T cells are allowed to undergo activation and expansion (12-14). The molecular mimicry mechanism is particularly interesting as a viral protein (U24) of both variant A and variant B of HHV-6 shares a stretch of 7 identical amino acids (residues 4-10) with human MBP (residues 96-102). This raises the possibility that MBP-reactive T cells may be susceptible to activation by HHV-6 containing this sequence homology (4,11,16,18). We have demonstrated recently that a significant proportion of $CD4^+$ and $CD8^+$ T cells that cross-react with both $HHV\text{-}6_{U24}$ and MBP can be detected in Chinese MS patients and that the frequency of cross-reactive T cells is increased in Chinese MS patients. Similarly, significant sequence homology between MBP (residues 83-99) and EBV (residues 627-641 of the DNA polymerase) has been reported. Some but not all MBP-reactive T cell clones recognizing the immunodominant region of MBP have been shown to cross-react with an EBV peptide containing this sequence homology. Molecular interactions between T cell receptor contact residues, the MBP peptide and the DRB1*1501 molecule, have been well studied (24). Recently, the crystal binding structure of both the EBV peptide and the MBP peptide to DR2 has been determined to provide a structural explanation for T cell cross-reactivity (22). It is important to further delineate whether these cross-reactive T cells are sensitized to undergo *in vivo* activation and expansion in MS patients, and whether $CD8^+$ cytotoxic cross-reactive T cells have a particular role in CNS demyelination through direct injury of oligodendrocytes (15). Both EBV and HHV-6 share extensive sequence homology with MBP and they may play an important role in the pathogenesis of MS by activating myelin autoreactive T cells through molecular mimicry. T cells cross-reactive with the viral proteins and MBP may be sensitized in MS patients as the viruses undergo active replication. $CD4^+$ and $CD8^+$ cross-reactive T cells induced by the viral antigens may mediate CNS pathology through distinct mechanisms.

First, this study has determined that $CD4^+$ T cells cross-reactive with EBV and MBP are sensitized in Chinese MS patients by measuring the precursor frequency of T cells cross-reactive with 15-mer synthetic peptides of EBV and MBP. The precursor frequency was determined in 12 Chinese MS patients and was analyzed in the context of HLA haplotypes and of cell-free serum or CSF HHV-6 DNA and EBV antibody status of the patients. Furthermore, we generated $CD4^+$ and $CD8^+$ cross-reactive T cell lines that we further characterized for functional recognition of the cross-reactive HHV-6, EBV and MBP peptides, and we showed that the binding of the peptide to the MHC groove is allele-dependent. We also showed that the HLA-DRB1* allele (DR4) represents the most dominant genotype (5/12) in Chinese MS patients. Significantly, there is a higher estimated frequency in the $DR4^+$-$CD4^+$T cell response group than in the non-$DR4^+$-$CD4^+$T response group. These results indicated that the $DR4^+$ allele molecule was able to bind the peptides of both $MBP_{81\text{-}95}$ and EBV. More importantly, there is a

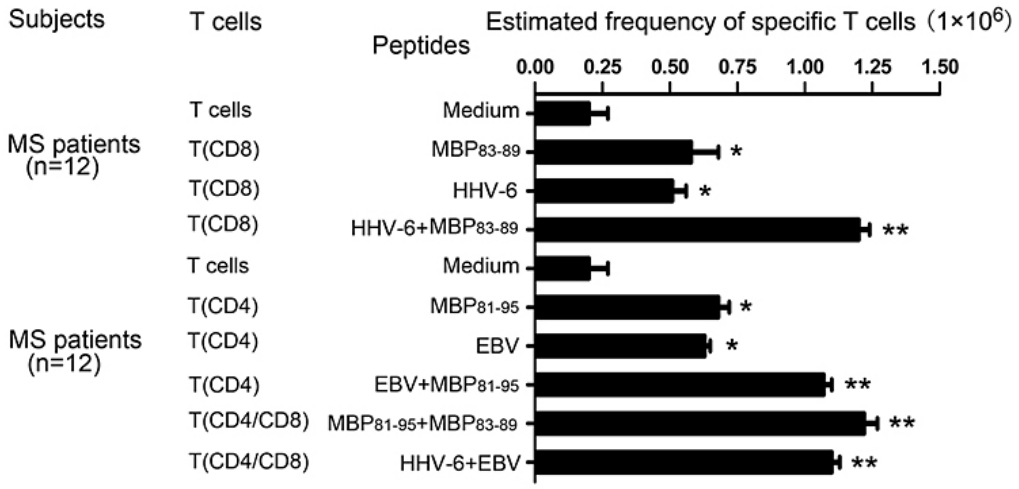

Figure 2. $CD4^+/CD8^+$T cell responses to the peptides corresponding to MBP and HHV-6/EBV in Chinese MS patients.

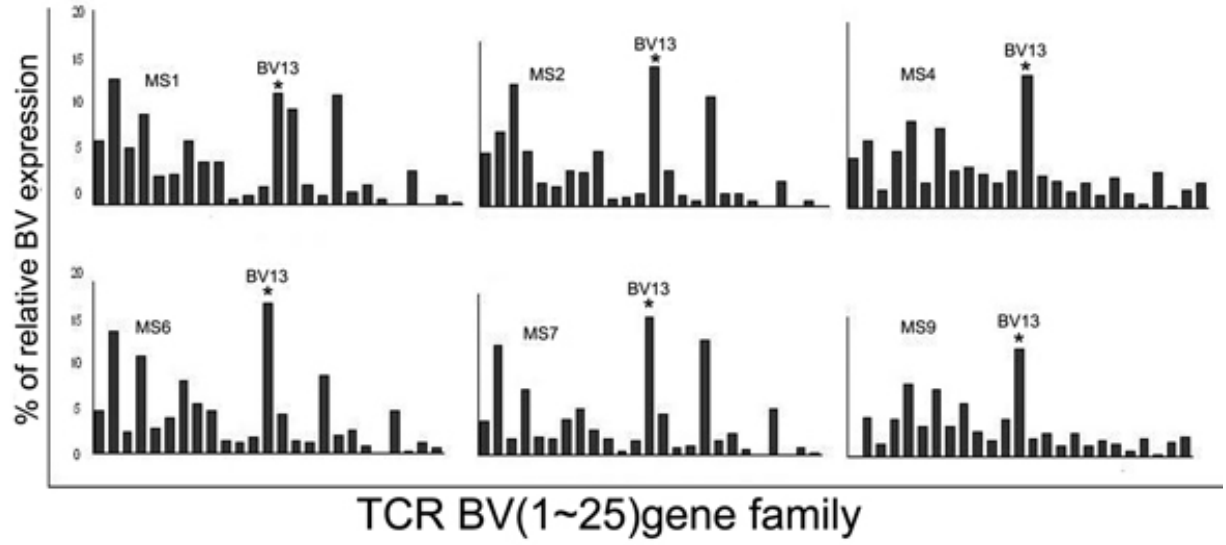

Figure 3. The reactivity pattern and TCR V gene usage of cross-reactive T cells. A series of real-time PCR analyses revealed a highly significant BV skewing to BV_{13} (8/13, 61.54%), BV_2 (5/13, 40.00%) and BV_4 (2/13, 15.40%) in T cell lines from MS patient.

closer relationship between $CD4^+$T cells and MHC-$DR4^+$ restriction in Chinese MS patients.

Our characterization has also confirmed that the CD8+ cross-reactive T cells recognizing MBP derived peptides are cytotoxic in nature. They recognize endogenously processed MBP and are able to damage human oligodendrocytes expressing appropriate HLA class I and MBP. This study has provided important evidence regarding the distinctive role of CD8+ cytotoxic cross-reactivity in CNS demyelination in Chinese MS patients. Also, we demonstrated that myelin-reactive T cells may be activated by microbial agents, in particular, viral antigens during the course of infection and re-activation through a mechanism known as molecular mimicry. This raises the possibility that MBP-reactive T cells may be susceptible to activation by HHV-6/EBV containing homologous sequence (25,26). We also have found that a significant proportion of CD4+ and CD8+ T cells that cross-react with both MBP83-89 /HHV-6 and MBP81-95/EBV can be detected in MS. These autoreactive T cells have a highly significant BV skewing for BV13 (8/13, 61.54%), BV2 (5/13) and BV4 (2/13) and the DRB1*04 genotype was the most dominant allele (5/12, 41.7%) compared to normal control group (28/150, 18.67%) in this cohort of Chinese MS patients.

Of particular note, our results suggest that, in contrast to CD4+ T cell counterparts, CD8+ cytotoxic T cells cross-reactive with HHV-6/EBV viral protein and MBP may contribute to demyelination by directly injuring oligodendrocytes expressing MHC class I/II molecules and

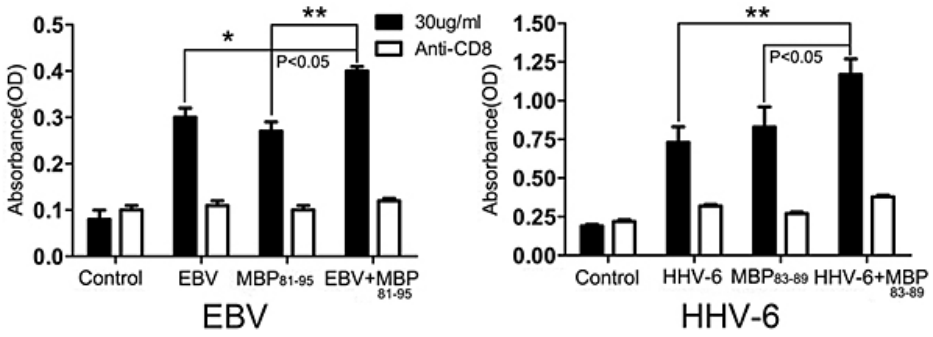

Figure 4. $CD8^+$ cytotoxic T cell line cross-reactivity between HHV-6 and MBP. $CD8^+$ T cell clones can be generated and characterized to study cross-reactivity and cytotoxicity. The cross-reactive $CD8^+$ T cells are cytotoxic towards autologous target cells pulsed with the MBP peptide in the context of HLA class I. $CD8^+$ cytotoxic T cell cross-reactivity between HHV-6 and MBP_{83-89} or EBV and MBP_{81-95}. The core sequence/epitope shared between MBP_{83-89} and HHV-6 or MBP_{81-95} and $EBV_{627-642}$ antigen may sensitize a subset of $CD8^+$ cytotoxic T cells that are able to cross-react with both antigens during the course of HHV-6 (Figure 4. $P<0.05$) or EBV (Figure 4. $P< 0.05$) re-activation in Chinese MS patients. These $CD8^+$ cytotoxic cross-reactive T cells may have significant pathological relevance in MS as they could directly induce injury of oligodendrocytes that are known to express both MBP and MHC class I molecules. It has been demonstrated that $CD8^+$ cytotoxic MBP-reactive T cells are able to cause CNS inflammation and extensive demyelination in MS, which closely resembles CNS lesions in the MS brain.

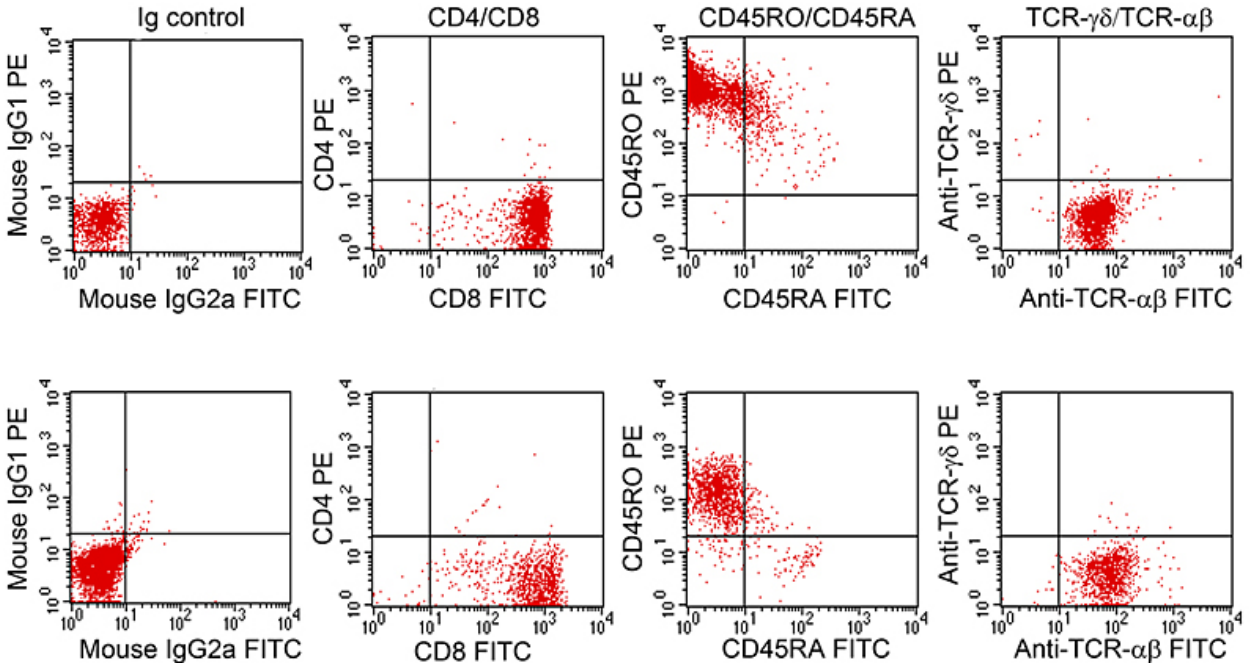

Figure 5. The phenotypic expression of the representative $CD8^+$ cross-reactive T cell lines. $CD8^+$ T cell lines were examined for the expression of CD4/CD8, CD45RA/CD45RO and TCRαβ/TCRγδ using monoclonal antibodies conjugated with FITC or PE by direct dual staining and flow cytometric analysis. Methods: 10^5 cells of each T cell line were washed in PBS containing 1% FBS and 0.1% sodium azide (FBS-PBS) and resuspended in 100 µl FBS-PBS containing fluorochrome-labeled antibodies (CD4/CD8, CD45RA/CD45RO, TCRαβ/TCRγδ, Becton Dickinson) or appropriate Ig isotype controls. After incubation for 30 min, the cells were washed three times in FBS-PBS, and analyzed using a FACS Calibur.

MBP. Therefore, there is a close relationship between HHV-6/EBV infection and the onset of demyelinating disease. The autoreactive T cells not only had a particular pattern of TCR BV gene usage, but were also restricted by MHC I/II in the MS patients (24,27-30). Importantly, the full scope of MS etiology and pathogenesis can be better understood when the mutual interactions between the immune and nervous systems are considered. Immuno-intervention or combination therapies could be helpful to block disease progression or repair damaged CNS tissues (31-34).

6. ACKNOWLEGMENTS

Weizhi Cheng, Yanhui Ma and Fang Gong contributed

Table 6. Restricted TCR BV gene usage in T cells lines derived from MS patients

No.	sex	age	BV 2	BV 4	BV 13
MS04	M	55	N	N	N
MS05	F	63	+	+	+
MS06	F	22	+	-	+
MS07	F	43	-	+	+
MS08	F	33	+	-	-
MS09	F	61	N	N	N
MS10	F	39	-	-	+
MS11	F	47	N	N	N
MS12	F	45	-	-	+
MS13	F	39	+	-	+
MS14	F	59	-	-	-
MS15	M	47	-	-	+
MS16	F	48	+	-	+

BV gene expression of each transcript was analyzed quantitatively by real-time PCR. BV gene distribution is presented as the mean % on the Y-axis. Student's t-test was used to calculate the statistical difference in BV2 (5/13), BV4 (2/13) and BV13 (8/13) expression in the MS patients, also as shown

Table 7. Preliminary characterization of CD8+ T cell Lines cross-reactive with HHV-6 and MBP

T cell line	Reactivity to (CPM ±SD)			% Specific cytotoxicity (E:T=10)			HLA-A.B. genotypes	
	Medium	HHV-6 peptide	MBP peptide	Control cells	HHV-6 cells	MBP cells	HLA-A.*	HLA –B *
MS1-E3	822±118	3391±198	2999±182	3.1 ±0.2	88.2 ± 3.6	65.9 ± 5.3	02,24	40,46
MS1-F2	1272±111	5411± 432	4872±322	5.4 ±0.4	64.1 ± 5.1	69.9 ± 3.7	11,24	13,46
MS2-E9	748±290	4532±229	3201±200	4.1 ±0.2	70.2 ± 3.7	85.8± 4.4	24,33	44,57/58
MS2-H2	788±270	5428±987	4441±278	8.8±0.5	55.6 ± 3.2	66.9 ± 7.1	02,11	15,45
MS3-G10	2433±167	9123±1549	7772±365	7.7 ±0.3	88.9± 9.	45.1 ± 2.2	02,24	15,38
MS3-B8	699±401	3881±222	1499±280	4.9±0.2	54.2 ± 1.9	65.3 ± 6.1	02,11	15,38

The selected T cell lines were representative and had high stimulation indices. T cell lines examined were found to cross-react with the MBP peptide that differs in two flanking residues from the HHV-6 peptide. All the obtained CD8+ T cell lines exhibited cytotoxic activity against autologous EBV transformed B cells pulsed with the HHV-6 peptide. The CD8+ cross-reactive T cell lines also lysed autologous EBV transformed B cells pulsed with the MBP peptide.

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7. REFERENCES

1. Tejada-Simon M.V., Y.C. Zang, D. Yang, J. Hong, S. Li, R.A. Singh, E. Van den Berg-Loonen, J.M. Killian, V.M. Rivera, and J.Z. Zhang: Aberrant T cell responses to myelin antigens during clinical exacerbation in patients with multiple sclerosis. Int Immunol 12, 1641-50 (2000)

2. Lucchinetti C, W. Brück, J. Parisi, B. Scheithauer, M. Rodriguez, H. Lassmann: Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol 47, 707-717 (2000)

3. Tejada-Simon M.V., J. Hong, V.M. Rivera, and J.Z. Zhang: Reactivity pattern and cytokine profile of T cells primed by myelin peptides in multiple sclerosis and healthy individuals. Eur J Immunol, 31, 907-17 (2001)

4. Haahr S, P. Höllsberg: Multiple sclerosis is linked to Epstein-Barr virus infection. *Rev Med Virol* 16, 297-310 (2006)

5. Owens G.P., J.L. Bennett, D.H. Gilden and M.P. Burgoon: The B cell response in multiple sclerosis. *Neurol Res* 28, 236-244 (2006)

6. Cross A.H., J.L. Trotter and J. Lyons: B cells and antibodies in CNS demyelinating disease. *J Neuroimmunol* 112, 1-14 (2001)

7. Ziemssen T. and F. Ziemssen: The role of the humoral immune system in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). *Autoimmun Rev* 4, 460-467 (2005)

8. Sospedra M. and R. Martin: Immunology of multiple sclerosis. *Annu Rev Immunol* 23, 683-747 (2005)

9. Tejada-Simon M.V., Y.C. Zang, J. Hong, V.M. Rivera, J.M. Killian and J.Z. Zhang: Detection of viral DNA and immune responses to the human herpesvirus 6 101-kilodalton virion protein in patients with multiple sclerosis and in controls. *J Virol* 76, 6147-6154 (2002)

10.Tejada-Simon M.V., J. Hong, V.M. Rivera and J.Z. Zhang: Skewed autoantibody reactivity to the extracellular domain of myelin oligodendrocyte glycoprotein in multiple sclerosis. *Immunology* 107, 403-410 (2002)

11.Tejada-Simon M.V., Y.C. Zang, J. Hong, V.M. Rivera and J.Z. Zhang: Cross-reactivity with myelin basic protein

and human herpesvirus-6 in multiple sclerosis. *Ann Neurol* 53, 189-197 (2003)

12.Sun W., H. Nie, N. Li, Y. C. Zang, D. Zhang, G. Feng, L. Ni, R. Xu, S. Prasad, R. R. Robinson, W. Ho, E. Sercarz and J. Z. Zhang: Skewed T-cell receptor BV14 and BV16 expression and shared CDR3 sequence and common sequence motifs in synovial T cells of rheumatoid arthritis. *Genes Immun* 6, 248-261 (2005)

13.Chen G., N. Li, Y. C. Zang, D. Zhang, D. He, G. Feng, L. Ni, R. Xu, L. Wang, B. Shen and J. Z. Zhang: Vaccination with selected synovial T cells in rheumatoid arthritis. *Arthritis Rheum* 56, 453-463 (2007)

14.Xu G., H. Nie, N. Li, W. Zheng, D. Zhang, G. Feng, L. Ni, R. Xu, J. Hong and J. Z. Zhang: Role of osteopontin in amplification and perpetuation of rheumatoid synovitis. *J Clin Invest* 115, 1060-1067 (2005)

15.Zang Y.C., S. Li, V.M. Rivera, J. Hong, R.R. Robinson, W.T. Breitbach, J. Killian and J.Z. Zhang: Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. *J Immunol* 172, 5120-5127 (2004)

16. Cirone M., L. Cuomo, C. Zompetta, S. Ruggieri, L. Frati, A. Faggioni and G. Ragona: Human herpesvirus 6 and multiple sclerosis: a study of T cell cross-reactivity to viral and myelin basic protein antigens. *J Med Virol* 68, 268-272 (2002)

17. Hong J., Y. C. Zang, S. Li, V. M. Rivera and J. Z. Zhang: Ex vivo detection of myelin basic protein-reactive T cells in multiple sclerosis and controls using specific TCR oligonucleotide probes. *Eur J Immunol* 34, 870-881 (2004)

18.Wandinger K, W. Jabs, Siekhaus A, Bubel S, Trillenberg P, Wagner H, Wessel K, Kirchner H, Hennig H. MS.: Association between clinical disease activity and Epstein-Barr virus reactivation in MS. *Neurology* 55, 178-184 (2000)

19. Zang Y.C., J. Hong, V.M. Rivera, J. Killian and J.Z. Zhang: Human anti-idiotypic T cells induced by TCR peptides corresponding to a common CDR3 sequence motif in myelin basic protein-reactive T cells. *Int Immunol* 15, 1073-1080 (2003)

20. Huseby E.S., D. Liggitt, T. Brabb, B. Schnabel, C. Ohlen and J. Goverman: A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med* 194, 669-676 (2001)

21. Goverman J., A. Woods, L. Larson, L.P. Weiner, L. Hood, D.M. Zaller: Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* 72, 551-560 (1993)

22. Lang H.L., H. Jacobsen, S. Ikemizu, C. Andersson, K. Harlos, L. Madsen, P. Hjorth, L. Sondergaard, A. Svejgaard, K. Wucherpfennig, D. I. Stuart, J. I. Bell, E. Y. Jones and L. Fugger: A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nat Immunol* 3, 940-943 (2002)

23. Wang B., K. Yao, G. Liu, F. Xie, F. Zhou and Y. Chen: Computational prediction and identification of Epstein-Barr virus latent membrane protein 2A antigen-specific CD8+ T-cell epitopes. *Cell Mol Immunol*, 6, 97-103 (2009)

24. Sellebjerg F., J. Jensen, H. O. Madsen and A. Svejgaard: HLA DRB1*1501 and intrathecal inflammation in multiple sclerosis. *Tissue Antigens* 55, 312-318 (2000)

25. Fujinami R.S., M.G. von Herrath, U. Christen and J.L. Whitton: Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease. *Clin Microbiol Rev*, 19, 80-94 (2006)

26. Lehmann P.V., T. Forsthuber, A. Miller and E.E. Sercarz: Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358, 155-157 (1992)

27. Cabarrocas J., J. Bauer, E. Piaggio, R. Liblau and H. Lassmann: Effective and selective immune surveillance of the brain by MHC class I-restricted cytotoxic T lymphocytes. *Eur J Immunol* 33, 1174-1182 (2003)

28. Ridgway, W.M., H. Ito, M. Fassò, C. Yu and C.G. Fathman: Analysis of the role of variation of major histocompatibility complex class II expression on nonobese diabetic (NOD) peripheral T cell response. *J Exp Med* 188, 2267-2275 (1998)

29. Matsuoka T, H. Tabata, S. Matsushita: Monocytes are differentially activated through HLA-DR, -DQ, and -DP molecules via mitogen-activated protein kinases. *J Immunol* 166, 2202-2208 (2001)

30. Fogdell-Hahn A., A. Ligers, M. Gronning, J. Hillert and O. Olerup: Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease. *Tissue Antigens* 55, 140-148 (2000)

31. Zhang J.: T cell vaccination as an immunotherapy for autoimmune diseases. *Cell Mol Immunol* 1, 321-327 (2004)

32. Chen Y., H. Sun, G. Liu, B. Wang, F. Wang, B. Sun and K. Yao: EBV LMP2A-specific T cell immune responses elicited by dendritic cells loaded with LMP2A protein. *Cell Mol Immunol* 6, 269-276 (2009)

33. Guo L., Y. Li, H. Lin, X. Ji, J. Li, L. Que, Y. Zhang, Y. Rong and J. Wang: Evaluation of a rat model of experimental autoimmune encephalomyelitis with human MBP as antigen. *Cell Mol Immunol* 1, 387-391 (2004)

34. Qin Y., D.Q. Zhang, A. Prat, S. Pouly and J. Antel: Characterization of T cell lines derived from glatiramer-acetate-treated multiple sclerosis patients. *J Neuroimmunol* 108, 201-206 (2000)

Abbreviations: HHV-6: Human Herpes Virus 6; CSF: cerebrospinal fluid; MS: Multiple Sclerosis; OND: Other Neurologic Diseases; MBP: Myelin Basic Protein; EBV: Epstein-Barr Virus; PBMC: Peripheral Blood Mononuclear Cell; VCA: Viral Capsid Antigen; HLA: Human Leukocyte Antigen; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; TCR: T Cell Receptor; CD: Cluster of Differentiation; FBS: Fetal Bovine Serum; PBS: Phosphate Buffered Saline; MHC: Major Histocompatibility Complex

Key Words: Molecular mimicry, Multiple sclerosis, Human hepersvirus-6, Epstein-barr virus, Autoreactive T cell, $CD8^+$ T cell

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