Article

Analysis of the Conservation of T Cell Receptor Alpha and Beta Chain Variable Regions Gene in pp65 Peptide-Specific HLA-A*0201-Restricted CD8$^+$ T Cells

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Many viral epitope specific T cell receptors (TCRs) in MHC-matched individuals have been demonstrated to involve conserved amino acid motifs in β chain complementarity-determining region 3 (CDR3). However, it is not sure whether the conserved motifs can also be found in TCR α chain. In previous studies, we developed a modified method to enlarge the percentage of cytomegalovirus (CMV) pp65 peptide-specific CD8$^+$ T cells in PBMC by continuous peptide stimulation *in vitro*, which provides sufficient number of specific T cells for detection. In this study, we further analyzed the restrictive usage of TCR Vα and Vβ gene families and investigated the CDR3 gene sequence of pp65 peptide-specific CD8$^+$ T cells. Analysis of CDR3 spectratypes suggested a restricted usage of TCR α chain AV8, AV12, AV21, AV31 families and TCR β chain BV3, BV14, BV21, BV23, BV11 families in donor CD8$^+$ T cells stimulated by pp65 peptide. The sequences of these T cells involved similar sequence (TX) G (X) A in CDR3 region of TCR α chain and L (XT) G (X) A in TCR β chain. *Cellular & Molecular Immunology*. 2009;6(2):105-110.

Key Words: TCR, CDR3, CMV, pp65 peptide, CTL

Introduction

Cytomegalovirus (CMV) is a ubiquitous and infectious pathogen. CMV infection does not cause any disease and show any clinical symptoms in an immunocomplement body, but does in immunodeficiency or immunocompromised body, such as patients with acquired immune deficiency syndrome (AIDS) or organ transplant recipients. It can lead to inflammatory reactions mainly in genitourinary system, central nervous system and liver, and even develop severe deficiency in the immune system or death (1).

It has been reported that there are clonal expansions of certain T cell subgroups in the peripheral blood of CMV infectors in the acute phase (2). These T cells are considered as CMV-specific cytotoxic T lymphocytes (CTL), which play an important role in inhibiting CMV infection and re-activation (3). CMV-specific T cells can control the virus in the body with normal immune function. Once organism immune function is compromised, the balance will be broken, and then the virus will re-activate and cause disease. Treatment of CMV infectors with immunosuppressor would lead to CMV re-activation and be life-threatening (4).

CMV-specific T lymphocytes recognize CMV antigens by T cell receptor (TCR), which is composed of double-strand α and β. The key sites that combine peptide on TCR α and β chain are the complementarity determining region 3 (CDR3), which play an important role in identifying CMV peptide. The specific T cells will proliferate and form clonal populations when recognize the CMV peptide. Each T cell clone has its unique TCR CDR3, so analyzing the frequency of TCR CDR3 spectratypes can reflect the clonality of T cell and then help to find CMV-specific T cells.

Analyzing CMV-specific TCR α and β chain sequence to discover sharing of CMV-specific TCR among individuals is

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Abbreviations: TCR, T-cell receptor; CDR3, complementarity-determining region 3; CTL, cytotoxic T lymphocyte; PBMCs, peripheral blood mononuclear cells; CMV, cytomegalovirus; HLA, human leucocyte antigen; FACS, fluorescence activated cell sorting; BV, β chain variable gene segment; BD, β chain diversity gene segment; BJ, β chain joining gene segment; N, N-region; AV, α chain variable gene segment; AJ, α chain joining gene segment.

valuable for developing new approaches to diagnose CMV infection. In general, however, the pool sizes of the specific T cells in patient's body are very small. It is difficult to identify and detect these CMV reactive lymphocytes. To overcome this difficulty, we used a CMV phosphoprotein epitope $pp65_{495-503}$ peptide (sequence: NLVPMVATV), which has been shown to be immunodominant, stimulating peripheral blood mononuclear cells (PBMCs) to enlarge the percentage of antigen-specific CD8$^+$ T cells *in vitro* (5). The sequences of TCR CDR3 of CD8$^+$ T cell specific to CMV pp65 epitope peptide have been analyzed. Among three unrelated donors who had the same MHC class I allele of HLA-A*0201, the remarkable similarities have been found.

Materials and Methods

Subjects and cells

Three healthy donors included one man and two women, with an average age of 30 years old, ranging from 24 to 36 years old. The MHC class I tissue type of each donor was determined using serological typing Lymphocyte ABC-120 (Biotest Diagnostics, USA). PBMCs were isolated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation, and washed twice with RPMI-1640 medium then enriched for T cells by passing through nylon wool columns.

Magnetic bead separation of CD8$^+$ T cells

T cells were incubated with anti-CD8 monoclonal antibody coupled with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then loaded onto MidiMACS columns (Miltenyi Biotec) and CD8$^+$ T cells were positively selected according to the manufacturer's instructions. The separated CD8$^+$ T cells was detected by flow cytometry and the purity > 90% (data not shown).

Amplification of CMV-specific CTL

The peptides of HCMV pp65 (NLVPMVATV and RPPIFIRRL) provided by Guangzhou Taimo Biotechnology Limited Company were used, and both of them are restricted through HLA-A2. CD8$^+$ T cells (1×10^6) were resuspended in 100 μl of RPMI-1640 containing 10% fetal calf serum (FCS) and incubated with 50 μg/ml NLVPMVATV peptide at 37°C. At the same time, parallel studies were performed with the RPPIFIRRL peptide or with no peptide as matched controls. After 2 h, 2 ml of RPMI-1640 containing 10% FCS was added to each sample and the samples were cultured in 12-well plates at 37°C in a humidified 5% CO_2 incubator. On the second day, IL-2 was added to a final concentration of 100 U/ml. On the ninth day, 800 μl of the old medium was aspirated from each well and replaced with 1 ml of RPMI-1640 plus 10% FCS containing 100 U/ml of IL-2 and continue to cultivate. On the fifteenth day, an aliquot of the cells was aspirated to detect CMV-specific CTL by fluorescence-activated cell sorter (FACS) analysis. After the samples had been aspirated, the contents of the wells were replenished with fresh complete medium containing 100 U/ml IL-2. The experiments were repeated at least three times with similar results.

Flow cytometric analyses

CD8$^+$ T cells were aspirated from the wells and centrifuged at 300 × g for 5 min. Cells were washed twice with RPMI-1640 medium before cell count. CD8$^+$ T cells (2×10^5) were resuspended in 50 μl of cold FACS buffer (FB, 2% FCS and 0.1% sodium azide in PBS). Peptides MHC class I tetramers of HLA-A2 containing the HCMV pp65 peptides conjugated to PE were added at the appropriate dilutions. After incubation at 4°C in the dark for 1 h, the cells were washed twice with cold FB and fixed in PBS containing 2% formaldehyde before they were subjected to FACS analysis. Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (both from Becton Dickinson).

RNA extraction and cDNA synthesis

Total RNA was extracted from CD8$^+$ T cells (2×10^6/ samples) by RNA extracted kit (Omega Biotech Company, USA). Total RNA (1 μg) was reverse transcribed with 250 pm oligo (dT), 200 U MMLV reverse transcripatase (cDNA synthesis kit, MBI, Fermentas, USA), 250 μM of each dNTP in a total volum of 20 μl as recommended by the manufacturer. The cDNA was stored at -80°C before use as the template for PCR amplification.

PCR amplification of cDNA

The primers used for TCR BV gene family-specific amplification were consulting from Ref 6. CDR3 size analysis within the TCR α chain was performed by semi-nested PCR. Separate first-round PCR amplification reactions were performed for each of the 32 human AV gene families. These were carried out in a volume of 50 μl containing 2 μl each of the forward AV primer and the reverse AC primer, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, 200 mM of each dNTP, and 1 μl of cDNA. Following an initial denaturing step at 95°C for 5 min, PCR was carried out with 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s, with a final extension at 72°C for 10 min. An aliquot of 8 μl of each PCR product was electrophoresis on a 1.5% agarose gel and analyzed by ethidium bromide staining. Separate second-round PCR amplifications for each of the 32 human AV gene families were carried out in 25 μl volume containing 2 μl each of the forward AV primer and the reverse AC-FAM primer, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, and 200 mM of each dNTP, with 4 μl of the first-round products as the template. Four cycles of denaturing at 95°C for 2 min, annealing at 60°C for 2 min, 72°C for 2 min, were performed, followed by a final extension at 72°C for 10 min.

PCR amplifications of TCR β chain were carried out in a volume of 50 μl containing 2 μl each of the 5' BV primer and the 3' BC primer, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, 200 mM of each dNTP, and 1 μl of cDNA. Following an initial denaturing step at 94°C for 3 min, PCR was carried out with 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C

for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. An aliquot of 8 μl of each PCR product was electrophoresis on a 2% agarose gel and analyzed by ethidium bromide staining and visualized under UV light.

Analysis of CDR3 spectratype by GeneScan

Fluorescent PCR products (2 μl) were mixed with 2 μl of formamide, 0.5 μl of loading dye (25 mM EDTA, 50 ng/ml blue dextran), 0.5 μl of GeneScan-500 TAMRA dye-labeled size standards. The mixture was denatured at 95°C for 2 min, and 2 μl was loaded onto the gel (6% acrylamide, 6 M urea sequencing gel) and run for 2 h on 373A DNA sequencer (Applied Biosystems, USA). DNA products of the appropriate lengths were analyzed using GeneScan software version 672 (7). The relative fluorescence intensity (RI) was measured as: RI (%) = 100 × (clonal peak area) / (total peak area). The following criteria were used to determine whether a given TCR BV family was clonal expansion: a single peak with an RI greater than 35%, twin peaks with each peak having an RI greater than 25%, a skewed distribution where the peak RI of the skewed family was greater than 25%.

Sequencing the CDR3 of TCR AV and BV families showing restricted usage

The monoclonal expansion PCR products were electrophoresed and purified by gel, and amplification PCR again by the same TCR BV sense primers and TCR BC antisense primers (without FAM-labeled) at the same PCR condition, the PCR products purified and sequenced on an ABI 377 DNA sequencer (Invitrogen, ShangHai, China).

Results

Detection of CMV pp65 peptide-specific CTL

The flow cytometric analysis showed the percentage of CMV-specific CTL reached 67.76% after stimulation by 50 μg/ml NLVPMVATV peptides for 15 days. The percentage of CMV-specific CTL is 0.33% without peptide stimulation, and 1.49% with unrelated control peptide stimulation. The percentage of CMV-specific CTL in group stimulated by 50 μg/ml CMV-specific peptide is much higher than that without peptide stimulation or unrelated control peptide stimulation (Figure 1).

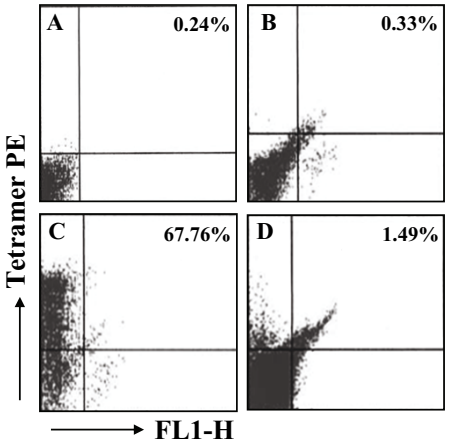

Figure 1. FACS detection of CD8$^+$ T cells of donor-1 after peptide stimulation for fifteen days. (A) Isotype-matched control. (B) Without the addition of peptide. (C) pp65 NLVPMVATV peptide stimulation (50 μg/ml). (D) Unrelated RPPIFIRRL peptide stimulation (50 μg/ml). The percentages of CMV-specific CTL were indicated.

TCR CDR3 spectratypes of CMV pp65 peptide-specific CTL

The RT-PCR products were analyzed on agarose gel by ethidium bromide staining and on polyacrylamide sequencing gel by fluorescence. The RT-PCR products of every TCR AV and BV gene family in both without peptide group and unrelated peptide stimulation group have a common specific band of about 250 bp when detected in agarose gels. However, this specific band could not be seen for several AV and BV gene families in the group stimulated by pp65 peptide. Each of these bands actually consists of several bands differing in length by 3 bp that can be separated in a 6% polyacrylamide gel. Approximately eight bands can be seen for all of TCR AV and BV gene families in two control groups. However, in group of pp65 peptide stimulation, fewer than eight bands or even a single band were observed for several TCR AV and BV gene families. The representative donor-1 BV families' fluorescent RT-PCR results were shown in Figure 2.

When comparing the CDR3 length distribution in two control groups and pp65 peptide stimulation group, we observed that the distribution in both two control groups showed a high degree of polymorphisms, about eight peaks can be observed in every TCR AV and BV gene family. A representative result of CDR3 length distribution for the TCR BV14 family in CD8$^+$ T cells stimulated by unrelated peptide was shown in Figure 2E. The distribution meant a polyclonal CDR3 pattern. In pp65 peptide stimulation group, however, an abnormal distribution with several peaks fewer than eight or even a single peak and skewed distribution of CDR3 spectratypes has been found in certain TCR BV families. In other words, an even more restricted CDR3 profile in pp65 peptide stimulating group has been detected. The CDR3 distribution showed different degrees of restriction.

The representative distribution of the TCR BV14 family CDR3 length for donor-1 stimulated by pp65 peptide was shown in Figure 2F. It can be seen that these T cells' CDR3-length polymorphism meant a monoclonal CDR3 pattern. Analysis of the CDR3 length distribution of T cell populations in pp65 peptide stimulation group revealed a restricted usage of TCR BV3, BV14, BV21, BV23, BV11 and TCR AV8, AV12, AV21, AV31 families.

Sequence analysis of the TCR CDR3 region

Those PCR products showing evidence of clonal expansion by GeneScan analysis were selected for CDR3 sequencing. The results showed a high similarity of N nucleotide insertion in TCR α and β chains. Conserved amino acid

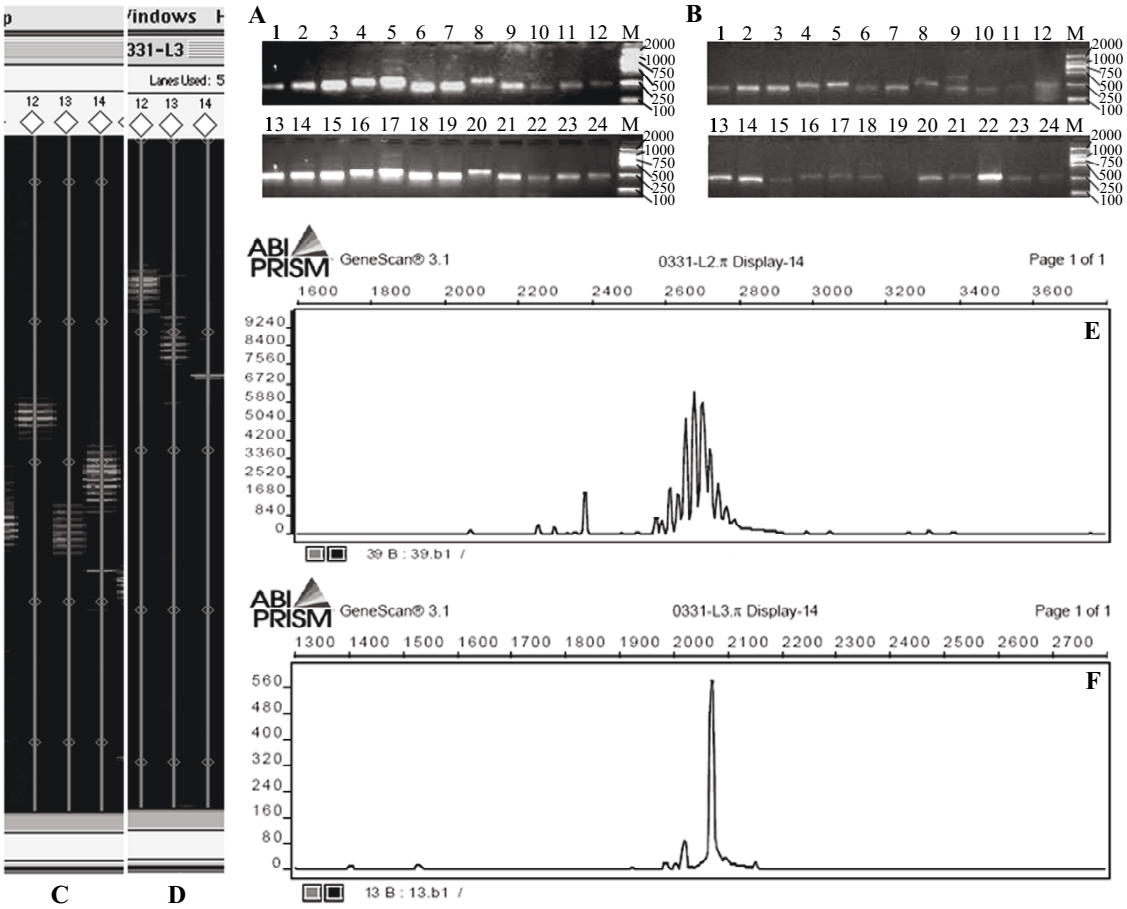

Figure 2. The GeneScan results of size and peak intensity of partial TCR BV CDR3 products of donor-1' CD8+ T cells after peptide stimulation. (A) The PCR products of TCR BV1-BV24 of donor-1' CD8+ T cells after unrelated peptide stimulation were analyzed on a 2% agarose gel by ethidium bromide staining. The numbers 1-24 denote the corresponding PCR products of TCR families BV1-BV24; (B) The PCR products of TCR BV1-BV24 of donor-1' CD8+ T cells after pp65 peptide stimulation; (C) Partially fluorescent PCR products of TCR BV of donor-1' CD8+ T cells after unrelated peptide stimulation analyzed on a 6% acrylamide sequencing gel; (D) Partially fluorescent PCR products of TCR BV of donor-1' CD8+ T cells after pp65 peptide stimulation; (E) CDR3 size and fluorescence intensity analysis of BV14 of donor-1' CD8+ T cells after unrelated peptide stimulation; (F) CDR3 size and fluorescence intensity analysis of BV14 of donor-1' CD8+ T cells after pp65 peptide stimulation.

motifs L (XT) G (X) A in TCR β chain (Table 1) and (TX) G (X) A in TCR α chain (Table 2) have been found in CD8+ T cells stimulated by pp65 peptide.

Discussion

CMV is a double-stranded DNA 230 kb β-herpesvirus, which if not been eradicated from body after primary infection, would retain a latent state within macrophage, granulocyte and dendritic cell precursors in host. When encountering viral antigens, CMV-specific CD8+ cytotoxic T cells undergo clonal expansion and exert immune function, playing an important role in control human CMV infection and immune protection from clinical CMV disease. It has been reported that the TCR repertoire of CD8+ T cells becomes more restrictive after primary human CMV infection, the restricted usage of distinctive TCR Vβ gene families are specific to certain CMV immunogenic agents (8). The restricted usage of those TCR Vβ gene families showed a conservative amino acid motif among unrelated HLA-matched infectors in their TCR β chain CDR3 region (9, 10). However, little information is available about restricted use of Vα genes in CMV-specific T cells. Whether TCR α chains also contain conservative CDR3 gene sequence is still unclear. The conservative motifs among HLA-matched individuals are often associated with the focused nature of TCRs recognizing a same epitope peptide (11).

CMV-specific CD8+ T cells are found at low frequency in peripheral blood lymphocytes of CMV-infecting patients, which only occupy 0.5-4% of CD8+ T cell pool. The low percentage of the CMV-specific CD8+ T cells in PBMC is not convenient to detection and analysis (12). To overcome this limitation, an established modified method with peptide stimulation to produce high percentage frequencies of

Table 1. The CDR3 gene and protein sequence of the monoclonal expansion CD8$^+$ T cells' TCR β chain in three donors

Samples	TCR β chain junktional sequence (CDR3)		
	TCR BV	BV N BD N BJ	TCR BJ
Donor-1	BV3	6 aa	BJ2S1
	GCC ATG TAC CTG TGT GCC AGC AGC	CCT CAC GGA GAA TTT TTC	AAT CAG GAC CAG CAC TTT GGC
	A M Y L C A S S	L (T) G A F F	N Q D Q H F G
Donor-1	BV14	6 aa	BJ2S7
	GCC ATG TAC CTG TGT GCC AGC AGC	CCT GGA CAC GGA GAA CTT	GAG CAG TAC TTT GGC
	A M Y L C A S S	L (G) (T) G A L	E Q Y F G
Donor-2	BV21	6 aa	BJ1S1
	TCT GTG TAC TTC TGT GCC ATC AGT	CCT CAC GGA CAC GAA AAT	GAG AAA CTC TTT TTC GGG
	S V Y F C A I S	L (H) G A N T	E K L F F G
Donor-2	BV23	5 aa	BJ1S1
	TCT GTG TAC TTC TGT GCC ATC AGT	CCT GGA GGA GAA CCG	GAG GAA TTT TTC GGG
	S V Y F C A I S	L (G) G A P	E A F F G
Donor-3	BV11	6 aa	BJ1S5
	GCC ATG TAC CTG TGT GCC AGC AGT	CCT GGA GGA CAG GAT GAA	AAT CAG CCG CAG CAC TTC GGG
	A M Y L C A S S	L (G) G (Q) (D) A	N Q P Q H F G

The amino acid with underline indicates the conserved sequence.

Table 2. The CDR3 gene and protein sequence of the monoclonal expansion CD8$^+$ T cells' TCR α chain in three donors

Samples	TCR α chain junktional sequence (CDR3)		
	TCR AV	AV N AJ	TCR AJ
Donor-1	AV8	5 aa	AJ44
	GCT GCT GTC TTC TGC GGT GCA GAA	ACA GGA GCT GGT AGC	AAA CTC ACC TTT GGC ACA GGA ACA AGA
	A A V F C G A E	(T) G A G S	K L T F G T G T R
Donor-1	AV12	5 aa	AJ29
	GCC GCT TAC TTC TGT GCC CTG AGC	AAG ACC GGA GCA CTT	AAT CTT ATC TTT GGA AAG GGC ACA AGA
	A A Y F C A L S	K (T) G A L	N L T F G K G T R
Donor-2	AV21	5 aa	AJ27
	TCT GTG TAC TTC TGT GCA GCA AGC	GCC GGA GCT ATC GAT	AAA CTC ACC TTC GGG GAT GGG ACT ACG
	S V Y F C A A S	(A) G A T D	K L T F G D G T T
Donor-2	AV12	5 aa	AJ42
	GCC GCT TAC TTC TGT TGT GCT AGT	ACC GGA GGA GCC AGA	AAT CTT ATC TTT GGA AAG GGC ACA AAA
	A A Y F C C A S	(T) (G) G A R	N L T F G K G T K
Donor-3	AV31	5 aa	AJ5
	GCC GTG TAC TAC TGT CTT CTG GGG	GCT GGA CTC GAT GCT	AAA CTT ACC TTT GGG AGT GGA ACA AGA
	A V Y Y C L L G	(A) G (L) (D) A	K L T F G S G T R

The amino acid with underline indicates the conserved sequence.

CMV-specific CTL has been used, which can be detected by tetramers and CDR3 spectratype analysis from a single blood sampling (5).

The TCR repertoire of CD8$^+$ T cells in donors' PBMCs is diverse without peptide stimulation, but rapidly becomes more restrictive after being stimulated by CMV pp65 peptide. Certain TCR AV and BV families' T cells are clonal expansion, which are considered to be CMV pp65 peptide specific. That is in agreement with a previous report which found common TCR BV families in CD8$^+$ T cells specific for the same peptide in human CMV carriers (13).

Results from some investigations in the recent years have provided evidence that virus-specific T cells show conservation of amino acid motifs in their TCR CDR3 regions among unrelated donors (8). Our results also showed that TCRs of CD8$^+$ T cells deriving from different donors but specific to the same CMV pp65 peptide have similar sequence L (XT) G (X) A in TCR β chain and (TX) G (X) A in TCR α chain CDR3 region. Since the CTL clones in the study are acquired from continuously stimulation by pp65 peptide, different from clones acquired from stimulation by antigen protein, these clones are single epitope specific. So these CTL clones from three donors should therefore have higher similarities and representation. The results suggesting

that the conserved motif may be linked with certain degree similarity of pp65 epitope stimulation among different individual.

The results provide a basis for applying the CDR3 spectratype analysis technology to aid the diagnosis and prognosis of clinical CMV infection associated disease. For example, by detecting the existence of a conservative motif can aid diagnose of CMV infection. However, much larger numbers of donors or patients are required in future studies.

Acknowledgements

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