Relationship between T-cell receptor beta chain sequences and human cytomegalovirus infection in allogeneic hematopoietic stem cell transplant recipients

ZHIHUA WU1,2, HUIPING ZHANG3, MIN JIN1, HANYING LIANG1, YAPING HUANG1, RONG YANG1, GENYONG GUI1, HUIQI WANG1, SHENGNAN GONG1, JINDONG WANG1 and JUN FAN1

1Virology Department, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003; 2Department of Clinical Laboratory, Tongde Hospital of Zhejiang Province, Hangzhou, Zhejiang 310012; 3Department of Clinical Laboratory, Hangzhou Cancer Hospital, Hangzhou, Zhejiang 310002, P.R. China

Received January 20, 2016; Accepted February 10, 2017

DOI: 10.3892/mmr.2017.6453

Abstract. In the present study, clonal amplifications of T-cell receptor β variable (TCR BV) linked to human cytomegalovirus (HCMV) infection were detected in recipients of allogeneic hematopoietic stem cell transplants (HSCT), and certain relationships between them were identified. Furthermore, the relationship between TCR BV sequences and HCMV infections was investigated. The results indicated that the 3 recipients of HSCT had monoclonal expansion of specific TCR BV clones following HSCT. Among these recipients, 2 suffered from pp65 and immediate early (IE) antigenemia. These patients demonstrated preferential expansion of TCR BV9 (QVRGGTDTQ) and TCR BV11 (VATDFQ). The remaining recipient did not express TCR BV9 and TCR BV11, nor did this individual have pp65 and IE antigenemia. These results suggest that expression of TCR BV9 and TCR BV11 may be associated with HCMV antigenemia, and may be involved in the immune response. The amino acid sequences ‘QVRGGTDTQ’ and ‘VATDFQ’ may be involved in HCMV reactivation in patients who have undergone HSCT. Assessment of the TCR BV families may provide valuable insight into HCMV pathogenesis and may aid in the diagnosis and therapy for HSCT recipients infected with HCMV.

Introduction

Human cytomegalovirus (HCMV) infection is a major cause of high morbidity and mortality in patients that have undergone allogeneic hematopoietic stem cell transplantation (HSCT) (1,2). Cellular immunity through antigen-specific cytotoxic T lymphocytes (CTLs) is involved in long-term suppression (3,4). Each individual CTL has a specific complementarity determining region 3 (CDR3) located in the T cell receptor β variable (TCR BV) region, which occurs as a result of V(D)J recombination and junctional diversity. During an antiviral immune response, the interactions between a TCR and its antigen-specific peptides, which are mediated in part by CDR3, result in a polyclonal expansion of T cells and clones expressing different CDR3 sequences (5). Determining the frequency of specific CDR3 sequences within a T-cell population may provide an accurate estimation of the extent of clonal expansion and the function of the expanded populations. It is well-known that TCRs are closely associated with viral infections, specifically hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (6,7). The occurrence of HCMV reactivation in patients following HSCT is also well documented (8,9). However, the underlying mechanisms responsible for this reactivation remain unknown. In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and DNA melting curve analysis were used to evaluate the distribution of TCR BV CDR3 genes expressed in peripheral blood mononuclear cells (PBMCs) isolated from patients that had undergone HSCT. This analysis evaluated the impact of T-cells on HCMV reactivation beyond T cell clonal expansion, thus providing molecular evidence that an association exists between HCMV infection and immune dysregulation in patients following HSCT.
Materials and methods

Subjects. A total of 3 HSCT recipients at The First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China) were enrolled in the present study between January 2011 and December 2012. One healthy donor from the same hospital was enrolled as a control, in December 2012. Patients with the following viral infections were excluded from this study: HIV, HBV, hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus, and Epstein-Barr virus. Recipient 1 was not infected with HCMV, while repeated HCMV reactivation occurred in recipients 2 and 3. Additional patient information is listed in Table I. The present study was approved by the Ethics Committee of the First Affiliated Hospital at the Medical School of Zhejiang University (Hangzhou, China). Written, informed consent was obtained from patients according to the Declaration of Helsinki.

TCR BV CDR3 genes expressed in PBMCs from all subjects were detected using RT-qPCR and a DNA melting curve analysis at the third month after transplantation (10,11). HCMV-pp65, HCMV-immediate early protein (HCMV-IE), HCMV-immunoglobulin (HCMV-Ig) M and HCMV-IgG were detected on the same day, and serial analysis of HCMV infection continued monthly until ~1 year after HSCT.

RNA extraction and cDNA synthesis. A total of 5 ml blood was collected from each subject, and PBMCs were isolated using a Ficoll-Paque density gradient technique. Total RNA was extracted using TRNzol reagent (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Sample RNA of 1-5 µg was reverse transcribed with the Oligo(dT18) primer in a 20 µl reaction volume and stored at -80°C prior to being used as the template for PCR amplification.

RT-qPCR amplification of CDR3 cDNA. In this study, we used primers for 24 TCR BV gene families (Table II) (12). A TransStart TM Green qPCR Super Mix (Tiangen Biotech Co., Ltd., Beijing, China) was used for qPCR. PCR reactions contained 0.5 µl reverse primer (TCR BV), 0.5 µl forward primer (24 TCR BV genes), 1 µl template cDNA, 12.5 µl qPCR Super Mix (2X), 0.5 µl passive reference (50X), and 10 µl RNase-free distilled water, to a final volume of 25 µl. Reactions were performed using an ABI 7500 system and were analyzed with v2.0.6 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction parameters were as follows: 2 min at 94°C to activate the GoTaq DNA polymerase enzyme (Promega Corporation, Madison, WI, USA), followed by 45 cycles at 94°C for 15 sec, 56.0°C for 25 sec, 72°C for 35 sec, 80°C for 2 sec, and a final extension at 72°C for 8 min. The melting step was performed by slow heating from 75 to 95°C with a ramping rate of 0.2°C/s, during which the fluorescence signal was continuously measured. Simultaneous amplification of TCR β chain constant 1 and glyceraldehyde 3-phosphate dehydrogenase were used as positive controls.

CDR3 sequencing of monoclonal TCR BV families. Using this melting curve, PCR products from the TCR BV gene families that had a single-peak expansion were selected. Single-peak expansion was defined as ‘monoclonal’, and appeared as only one main peak in the gene melting spectral pattern (GMSP). ‘Nonskewed’ means polyclonal amplification, which appeared as no visibly apparent main peak. The PCR products were re-amplified using GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA) by touchdown PCR. The parameters were as follows: pre-incubation at 95°C for 2 min, 95°C for 30 sec, 40.5°C for 40 sec and 72°C for 45 sec, and 6 cycles with annealing temperature decreasing 0.5°C per cycle, followed by 34 cycles of 95°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec. At the end, a terminal elongation step at 72°C for 8 min was added. Nested PCR products were sequenced using an ABI 3730 DNA Sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.). Samples were sent to Sangon Biotech Co., Ltd., Shanghai, China, and sequenced there. Results were analyzed automatically by a 3730xl DNA Analyzer and the sequencing reagent was BigDye terminator version 3.1.

Analysis of CDR3 sequences. Chromas software version 2.22 (Technelysium Pty Ltd., Brisbane, Australia) was used to translate nucleotide sequences into amino acid sequences (13). When the PCR nucleic acid product underwent electrophoresis and only one band was present, this represented direct sequencing. The presence of a thin strip in addition to a clear band, suggested cloned sequencing. Samples were sent to Sangon Biotech Co., Ltd., and sequenced there.

Detection of HCMV-pp65 and IE antigenemia. Peripheral blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-anticoagulant tubes. To evaluate pp65 and IE antigenemia a standard two-step immunohistochemical method was used, as described previously (14,15). Briefly, 5x10⁴ PBMCs were fixed on polyclonal-coated slides, and incubated with mouse anti-HCMV (pp65 catalog no. ab53495; IE catalog no. ab53489; 1:100; Abcam, Cambridge, UK) monoclonal antibodies at 37°C for 30 min, and horseradish peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (catalog no. ab6728; 1:250; Abcam) at 37°C for 30 min. A total of 5x10⁴ PBMCs were fixed on one slide, and every sample was fixed on 2 slides. Results were quantified based on the average number of brown stained positive cells per 5x10⁴ leukocytes in the 2 slides. Cells were observed under a light microscope (BH-2; Olympus Corporation, Tokyo, Japan) with magnification x100/200/400.

Detection of HCMV-IgG/IgM. Blood samples were collected in EDTA-anticoagulant tubes. HCMV-antibody serostatus (IgG and IgM) was determined using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocols (Dia.Pro Diagnostic Bioprobes s.r.l., Milan, Italy).

Statistical analysis. Differences in HCMV antigenemia and the presence of HCMV-IgG/IgM among the 3 HSCT recipients were examined using one-way analysis of variance followed by Tukey's test. Graphical analyses of results were generated using Prism 5 (Graph-Pad, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Frequency of skewed TCR BV gene families and CDR3 sequences derived from monoclonal TCR BV expansion in 3 recipients of HSCT. All 3 recipients of HSCT demonstrated preferential expansion of specific TCR BV gene families. The characteristics of TCRBV CDR3 expression are visualized in Fig. 1. For recipient 1, 5 monoclonal peaks were observed and TCR BV5.1 was preferentially expressed. In recipient 2, 4 monoclonal peaks were observed, including TCR BV9, TCR BV11, and TCR BV17. In recipient 3, 10 monoclonal peaks were observed and TCR BV9, TCR BV11, and TCR BV21 were selectively expressed. Recipients 2 and 3 had 2 gene families in common; TCR BV9 and TCR BV11 (Fig. 1).

A single peak in a GMSP indicates monoclonal expansion of a particular TCR BV clone, and this was verified by direct sequencing. Representative amino acid sequences of the TCR BV CDR3 in PBMCs from all recipients are listed in Table III. All recipients had a CDR3 sequence length of 5-12 amino acids. The amino acid sequences of TCR BV9 (QVRGGTDTQ) and TCR BV11 (VATDFQ) were similar between recipients 2 and 3. The healthy control expressed a non-skewed TCR BV repertoire. Representative GMSPs for non-skewed and oligoclonally expanded TCRBV gene families are visualized in Fig. 2.

Detection of pp65 and IE antigenemia. In all transplant recipients, pp65 and IE antigenemia were monitored ~10 times for up to one year following HSCT. Samples obtained from recipient 1 were typically pp65- and IE-negative, while recipients 2 and 3 consistently tested positive for pp65 (Fig. 3A) and IE antigenemia (Fig. 3B). For recipient 2, the mean number of pp65-positive cells was 4.5/5x10⁴ PBMCs (range, 1-10 positive cells/5x10⁴ PBMCs), and the mean number of IE-positive cells was 4.3/5x10⁴ PBMCs (range, 1-9 positive cells/5x10⁴ PBMCs). For recipient 3, a mean number of pp65-positive cells of 4.6/5x10⁴ PBMCs (range, 2-8 positive cells/5x10⁴ PBMCs) and a mean number of IE-positive cells of 4.2/5x10⁴ PBMCs (range, 2-8 positive cells/5x10⁴ PBMCs) was observed.

Detection of HCMV serostatus (IgG and IgM). Over the course of 1 year following HSCT, HCMV-specific IgG and IgM were investigated on 10 separate occasions for all 3 recipients, during detection of pp65 and IE antigens. During the present study, all recipients remained HCMV-IgG-positive and HCMV-IgM-negative.

Analysis of HCMV-pp65 and -IE antigenemia levels. The levels of HCMV-pp65 and IE antigenemia were evaluated using a standard two-step immunohistochemical method on 60 samples from 3 recipients following HSCT. All recipients were tested on 10 separate occasions, up to one year following HSCT and all data of HCMV-pp65 or HCMV-IE collected during this period were involved. Statistically significant differences were observed in the levels of HCMV-pp65 (P<0.05; Fig. 3A) and -IE (P<0.05; Fig. 3B) antigenemia between recipients 1 and 2. This result was also observed between recipients 1 and 3 (P<0.05 and P<0.05, respectively; Fig. 3A and B, respectively).

Table I. Characteristics of recipients and the healthy control.

<table>
<thead>
<tr>
<th>General information</th>
<th>Recipient 1</th>
<th>Recipient 2</th>
<th>Recipient 3</th>
<th>Healthy control</th>
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<td>20</td>
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<tr>
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<td>-</td>
</tr>
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<td>-</td>
</tr>
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<td>+/ +</td>
<td>+/ +</td>
<td>+/ +</td>
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<td>BUCY+MeCCNU</td>
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<tr>
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<tr>
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<td>GCV, ACV</td>
<td>GCV, ACV</td>
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</tr>
</tbody>
</table>

CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; AMMOL, acute myelomonocytic leukemia; HLA, human leukocyte antigen; GCV, ganciclovir; ACV, acyclovir; CSA, cyclosporin A; MMF, mycophenolate mofetil; PRED, prednisone; ARA-C, cytosine arabinoside; BU, busulfan; CY, cyclophosphamide; MeCCNU, methyl-cyclohexyl-nitrosamine; ATG, anti-thymocyte globulin; N/A, not applicable.
Comparison of HCMV antigenemia levels between TCR BV9/TCR BV11-positive recipients and the TCR BV9/TCR BV11-negative recipient. Recipients who preferentially expressed TCR BV9 and TCR BV11 were defined as TCR BV9+ and TCR BV11+, respectively. Among the 3 recipients enrolled, recipients 2 and 3 were TCR BV9+/TCR BV11+. ‘QVRGGTDQT’ was the conserved amino acid sequence in TCR BV9 CDR3 and ‘VATDFQ’ was observed in TCR BV11 CDR3 (Table III). Two recipients exhibited pp65 and IE antigenemia, while being simultaneously positive for HCMV-IgG and negative for HCMV-IgM. Recipient 1 was TCR BV9+/TCR BV11+, HCMV-IgG+ and HCMV-IgM+, but free of pp65 and IE antigenemia (Table IV). To determine whether HCMV reactivation was associated with a specific amino acid sequence of TCR BV CDR3, HCMV antigenemia status were compared between the TCR BV9+/TCR BV11+ recipients and the TCR BV9+/TCR BV11+ recipient (Table IV). This revealed that HCMV reactivation may be associated with TCR BV9 and TCR BV11, and a specific amino acid sequence of TCR BV CDR3 may be involved in HCMV infection.

Discussion

Although HCMV reactivation is commonly observed following immune dysfunction from HSCT, the molecular mechanisms that drive this phenomenon remain unknown. T cell immune responses induced by viral antigens are involved in the inflammatory process of stemming viral infections. CTLs are involved in HCMV control and pathogenesis (16). In PBMCs, >95% of the T cells are αβ+ (17). During T cell development, the TCR β chain undergoes rearrangement earlier than the α chain, according to rules of allelic exclusion. Therefore, analysis of the TCRBV CDR3 gene may be beneficial in determining the clonality of a particular T cell response, and therefore be used as a marker for the functional status of T cells (18). Studies focused on TCR BV gene families may provide novel insights and a solid foundation for the prevention, diagnosis, and treatment of viral infections (19).

The TCR BV gene family demonstrated a diverse, non-skewed expansion in PBMCs derived from the healthy control. However, particular TCR BV families were preferentially expressed or biased, and emerged as a group of monoclonal (oligoclonal) T cells in the PBMCs of all 3 recipients of HSCT. Due to the long-term use of systemic steroids, the T cell clonality of a particular T cell response, and therefore be used as a marker for the functional status of T cells (18). Studies focused on TCR BV gene families may provide novel insights and a solid foundation for the prevention, diagnosis, and treatment of viral infections (19).

Comparison of the HCMV serostatus (IgG and IgM) among recipients of HSCT. HCMV-specific-IgG and IgM was detected using ELISA on 60 samples from 3 patients 1 year following HSCT. Each recipient was tested 10 times on separate occasions. During the study, all recipients remained HCMV-specific-IgG-positive and IgM-negative. No significant difference between HCMV-IgG and IgM optical density/cut-off among the 3 recipients was observed. (P>0.05).
CDR3, and ‘VATDFQ’ was observed for TCR BV11 CDR3. The explanation for this finding may be the multifarious usage of distinctive TCR BV families. Conserved sequences of the TCR BV repertoire were diverse without peptide stimulation, but became more restrictive following stimulation by the HCMV peptide. The clinical course of HCMV reactivation was affected by the usage of TCR BV9 and TCR BV11 in recipients of HSCT. Expression of TCR BV9 and TCR BV11 may be associated with HCMV antigenemia, and may be involved in the immune response. The amino acid sequences ‘QVRGGTDTQ’ and ‘VATDFQ’ may be beneficial for eliciting an anti-viral response, as well as contributing to HCMV clearance. TCR BV with the sequences ‘QVRGGTDTQ’ and ‘VATDFQ’ may, therefore, be a risk factor for HCMV reactivation.

Although the number of patients that underwent HSCT enrolled in the present study was relatively small, each of the patients underwent detailed longitudinal analysis with 10 separate follow-ups over the course of 1 year. In total, >120 samples were analyzed. In addition, the contents of the present study are only a part of longer-term research work. Another challenge regarding the methodology is that the controls would have been improved had they been HCMV-negative patients that underwent HSCT. However, ~100% Han Chinese people
are HCMV-IgG positive (27), and HCMV-IgG negative HSCT recipients were not identified. Therefore, such negative controls were not used in the present study.

Given that immune reconstitution must start from the beginning for a patient who has undergone HSCT, these patients are at high risk for HCMV reactivation. The results from the present study provide a link between HCMV reactivation and immune homeostasis, and thus help to establish a prophylaxis and diagnosis of HCMV reactivation following HSCT. Assessment of clonal diversity of TCR against HCMV may provide important insights into the molecular basis of T cell immunodominance. However, further investigation is necessary to address this issue in recipients of HSCT.
Table IV. HCMV antigenemia status and TCR BV9/BV11 expression among the 3 recipients of allogeneic hematopoietic stem cell transplantation.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>TCR BV9</th>
<th>TCR BV11</th>
<th>HCMV-pp65</th>
<th>HCMV-IE</th>
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<tbody>
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<td>1</td>
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<td>2</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

TCR BV9/BV11*, recipients who preferentially expressed TCR BV9/BV11; TCR BV9/BV11, recipients who did not express TCR BV9/BV11; HCMV-pp65/IE*, recipients who exhibited pp65/IE antigenemia; HCMV-pp65/IE, recipients who were free of pp65/IE antigenemia. TCR BV9, T-cell receptor β variable; HCMV, human cytomegalovirus; IE, immediate early.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 30872239) and the Zhejiang Provincial Natural Science Foundation of China (grant no. LY14H100002).

References