難治性疾患等克服研究事業(免疫アレルギー疾患等予防・治療研究事業 移植医療研究分野)

「臓器移植・造血幹細胞移植後の日和見感染に対する有効かつ安全な多ウイルス特異的T細胞療法の開発と導入に関する研究」　(研究代表者　森尾友宏)

平成 26 年度　第 1 回造血細胞移植合同班会議

期日:平成 26 年 7 月 5 日(土)14 時 30 分～15 時 15 分
会場:名古屋第一赤十字病院　内ヶ島講堂

14:30～14:33　イントロダクション
東京医科歯科大学・発生発達病態学分野　森尾友宏

14:33～14:45　非ウイルスベクターを用いたキメラ抗原受容体遺伝子導入T細胞療法の開発
名古屋大学大学院医学系研究科・成長発達医学　高橋義行

14:45～14:57　多ウイルス特異的 T 細胞の機能評価
東京医科歯科大学・発生発達病態学分野　森尾友宏
東京医科歯科大学・発生発達病態学分野　小野敏明
東京大学医科学研究所　藤田由利子
東京大学医科学研究所　高橋　聡

14:57～15:09　ウイルス特異的 T 細胞のエピトープマッピングと HLA 拘束性の特定に関する研究
東京大学医科学研究所　立川　愛

15:09～15:15　多ウイルス特異的 T 細胞の臨床応用に向けて
東京医科歯科大学・発生発達病態学分野　森尾友宏

難治性疾患等克服研究事業(免疫アレルギー疾患等予防・治療研究事業 移植医療研究分野)

「臓器移植・造血幹細胞移植後の日和見感染に対する有効かつ安全な多ウイルス特異的T細胞療法の開発と導入に関する研究」　(研究代表者　森尾友宏)

平成 26 年度　第 2 回造血細胞移植合同班会議

期日:平成 27 年 1 月 10 日(土)10 時 00 分～10 時 45 分
会場:独立行政法人 国立がん研究センター中央病院　国際会議室・研究所セミナールーム

10:00 ～ 10:10　長期に凍結保存されたウイルス特異的CTLの安定性に関する検討
名古屋大学医学部附属病院 先端医療・臨床研究支援センター　西尾信博
名古屋大学大学院医学系研究科成長発達医学　小島勢二
名古屋大学大学院医学系研究科成長発達医学　高橋義行

10:10 ～ 10:20　実臨床応用に向けたウイルス特異的T細胞療法の開発
東京大学医科学研究所先端医療研究センター分子療法分野　藤田由利子
東京大学医科学研究所先端医療研究センター分子療法分野　田中ゆきえ

10:20 ～ 10:30　多ウイルス特異的T細胞におけるepitope mappingの検討
東京大学医科学研究所先端医療研究センター感染症分野　立川 愛
東京医科歯科大学発生発達病態学分野　小野敏明

10:30 ～ 10:40　多ウイルス特異的T細胞療法の臨床応用
東京医科歯科大学発生発達病態学分野・細胞治療センター　森尾友宏

10:40 ～ 10:45　総合討論

V　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Koura U, Sakaki-Nakatsubo H, Otsubo K, Nomura K, Oshima K, Ohara O, Wada T, Yachie A, Imai K, **Morio T**, Miyawaki T, Kanegane H.	Successful treatment of systemic cytomegalovirus infection in severe combined immunodeficiency using allogeneic bone marrow transplantation followed by adoptive immunotherapy.	J Investig Allergol Clin Immunol.	24	200-2	2014
Endo A, Watanabe K, Ohye T, Matsubara T, Shimizu N, Kurahashi H, Yoshikawa T, Katano H, Inoue N, Imai K, Takagi M, **Morio T**, Mizutani S.	Molecular and virological evidence of viral activation from chromosomally integrated HHV-6A in a patient with X-SCID.	Clin. Infect. Dis.	59	545-8	2014
Nakatani K, Imai K, Shigeno M, Sato H, Tezuka M, Okawa T, Mitsuiki N, Isoda T, Tomizawa D, Takagi M, Nagasawa M, Kajiwara M, Yamamoto M, Arai A, Miura O, Kamae C, Nakagawa N, Homma K, Nonoyama S, Mizutani S, **Morio T**.	Cord blood transplantation is associated with rapid B cell neogenesis compared with bone marrow transplantation.	Bone Marrow Transplant.	49	1155-61	2014
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Konuma T, Kato S, Ooi J, Oiwa-Monna M, Yuji K, Ohno N, Kawamata T, Jo N, Yokoyama K, Uchimaru K, Tojo A, **Takahashi S**.	Comparable long-term outcome of unrelated cord blood transplantation with related bone marrow or peripheral blood stem cell transplantation for patients aged 45 years or older with hematologic malignancies after myeloablative conditioning.	Biol Blood Marrow Transplant.	20	1150-5	2014
Nakaya A, Mori T, Tanaka M, Tomita N, Nakaseko C, Yano S, Fujisawa S, Sakamaki H, Aotsuka N, Yokota A, Kanda Y, Sakura T, Nanya Y, Saitoh T, Kanamori H, **Takahashi S**, Okamoto S.	Does the hematopoietic cell transplantation specific comorbidity index (HCT-CI) predict transplantation outcomes? A prospective multicenter validation study of the Kanto Study Group for Cell Therapy.	Biol Blood Marrow Transplant.	20	1553-9	2014
Takahashi S.	Here comes the cord.	Blood Res.	49	209-10	2014
Kawashima N, Ito Y, Sekiya Y, Narita A, Okuno Y, Muramatsu H, Irie M, Hama A, **Takahashi Y**, Kojima S.	Choreito formula for BK virus-associated hemorrhagic cystitis after allogeneic hematopoietic stem cell transplantation.	Biol Blood Marrow Transplant.	21	319-25	2015
Kobayashi R, Yabe H, Kikuchi A, Kudo K, Yoshida N, Watanabe K, Muramatsu H, **Takahashi Y**, Inoue M, Koh K, Inagaki J, Okamoto Y, Sakamaki H, Kawa K, Kato K, Suzuki R, Kojima S.	Bloodstream infection after stem cell transplantation in children with idiopathic aplastic anemia.	Biol Blood Marrow Transplant.	20	1145-9	2014

Ohye T, Inagaki H, Ihira M, Higashimoto Y, Kato K, Oikawa J, Yagasaki H, Niizuma T, **Takahashi Y**, Kojima S, Yoshikawa T, Kurahashi H.	Dual roles for the telomeric repeats in chromosomally integrated human herpesvirus-6.	Sci Rep.	4	4559	2014
Suzuki M, Ito Y, Shimada A, Saito M, Muramatsu H, Hama A, **Takahashi Y**, Kimura H, Kojima S.	Long-term parvovirus B19 infections with genetic drift after cord blood transplantation complicated by persistent CD4+ lymphocytopenia.	J Pediatr Hematol Oncol.	36	e65-8	2014
Gu L, **Kawana-Tachikawa A**, Shiino T, Nakamura H, Koga M, Kikuchi T, Adachi E, Koibuchi T, Ishida T, Gao GF, Matsushita M, Sugiura W, Iwamoto A, Hosoya N.	Development and Customization of a Color-Coded Microbeads-Based Assay for Drug Resistance in HIV-1 Reverse Transcriptase.	PLoS One.	9	e109823	2014
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VI　研究成果の刊行物・別刷

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Successful Treatment of Systemic Cytomegalovirus Infection in Severe Combined Immunodeficiency Using Allogeneic Bone Marrow Transplantation Followed by Adoptive Immunotherapy

U Koura,[1] H Sakaki-Nakatsubo,[1] K Otsubo,[1] K Nomura,[1] K Oshima,[2] O Ohara,[2,3] T Wada,[4] A Yachie,[4] K Imai,[5] T Morio,[6,7] T Miyawaki,[1] H Kanegane[1]

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Key words: Cytomegalovirus. Severe combined immunodeficiency. Bone marrow transplantation. Donor lymphocyte infusions.

Palabras clave: Citomegalovirus. Inmunodeficiencia combinada severa. Transplante de médula. Infusiones de linfocitos del donante.

Severe combined immunodeficiency (SCID) is one of the most severe forms of primary immunodeficiency disease. Although infants with SCID generally appear healthy at birth, they are unable to clear infections during the first few months of life. The presence of insidiously progressive respiratory disease with radiological evidence of interstitial pneumonia suggests the involvement of *Pneumocystis jiroveci* or cytomegalovirus (CMV) infection.

Hematopoietic stem cell transplantation (HSCT) and gene therapy are the curative treatments of choice for patients with SCID. Outcome is poor in those with ongoing *P jiroveci* or CMV infection. Adoptive immunotherapy with CMV-specific cytotoxic T lymphocytes (CTL) has recently been used for post-HSCT patients with refractory CMV infections [1,2]. However, this therapy is labor-intensive and expensive. Therefore, it is only used in a limited number of institutions. Conversely, activated $CD4^+$ T cells can be easily expanded ex vivo and used for adoptive immunotherapy against cancer or as a component of therapy based on donor lymphocyte infusion (DLI) [3-5]. Numazaki et al [6] reported the case of an infant diagnosed with severe interstitial pneumonia associated with CMV infection who was successfully treated with adoptive immunotherapy using activated $CD4^+$ T cells.

We report the case of a patient with X-linked SCID and systemic CMV infection. Treatment involved bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)–matched sibling donor and adoptive immunotherapy administered by infusion of CMV-positive donor-derived activated $CD4^+$ T cells. This combination successfully cured CMV infection.

A 3-month-old Japanese boy was admitted to a local hospital because of long-standing severe cough. The laboratory studies revealed lymphopenia, and the patient was transferred to Toyama University Hospital, Toyama, Japan owing to the possibility of primary immunodeficiency disease. Physical examination revealed fever, tachypnea, respiratory retraction, and hepatomegaly. The laboratory studies revealed lymphopenia (378/μL), thrombocytopenia (36 x 10^3/μL), anemia (hemoglobin, 7.7 g/dL), hypoproteinemia (total protein, 4.0 g/dL), elevated liver enzymes (aspartate aminotransferase, 564 IU/L; alanine aminotransferase, 91 IU/L; lactate dehydrogenase, 1088 IU/L), and hypogammaglobulinemia (IgG, 85 mg/dL; IgA, 1 mg/dL; IgM, 29 mg/dL). The common γ chain was not expressed on lymphocytes, and the genetic analysis revealed a novel 609delG mutation in *IL2RG*. Therefore, the patient was diagnosed with X-linked SCID. A chest radiograph showed interstitial shadow, and the thymus was not visible. Thoracic computed tomography images demonstrated consolidation in the right lower lobe and diffuse ground-glass opacities in both lungs; the thymus was not visible. Tests for *P jiroveci, Aspergillus* species, and *Candida* species were all negative. However, the test for CMV antigenemia was positive (C7-HRP, 34/44 000). In addition, CMV-DNA was detected in all the samples analyzed, including blood, urine, sputum, cerebrospinal fluid, and stool. Taken together, the data indicated systemic CMV infection.

The patient was immediately treated with intravenous immunoglobulin, ganciclovir, trimethoprim-sulfamethoxazole, and sivelestat sodium. However, a pulmonary hemorrhage and hypoventilation due to severe CMV pneumonia required mechanical ventilation. Although methylprednisolone pulse therapy and nitrogen oxide inhalation were also administered for CMV pneumonia, the patient's respiratory condition worsened. On the 13th day of hospitalization, the patient underwent BMT from an HLA-matched sibling without conditioning. Ciclosporin A and methylprednisolone were administered as prophylaxis for graft-versus-host disease.

The patient's respiratory condition gradually improved, and he was taken off the mechanical ventilator on day 8 after BMT (Figure). The donor was positive for anti-CMV antibody and had CMV-specific $CD8^+$ T cells (0.02%). Before BMT, activated $CD4^+$ T cells were prepared from the donor to use in the treatment of severe CMV infection. Although the patient was not under mechanical ventilation at this stage, he still required oxygen, and his CMV-DNA copy number was high. Therefore, he was treated with foscarnet beginning on day 18 after BMT for possible ganciclovir-resistant CMV infection. He also received CD4-DLI (5 x 10^7 cells) on days 24 and 38 after BMT. CMV-DNAemia had disappeared by day 39 after BMT. Although the patient has a mild developmental delay, he is doing well without intravenous immunoglobulin (replacement).

Various types of infection have been observed in patients with SCID. One of the major problems affecting these patients is interstitial pneumonia due to *P jiroveci* or CMV infection. CMV-induced pneumonia may be more severe and leads to a

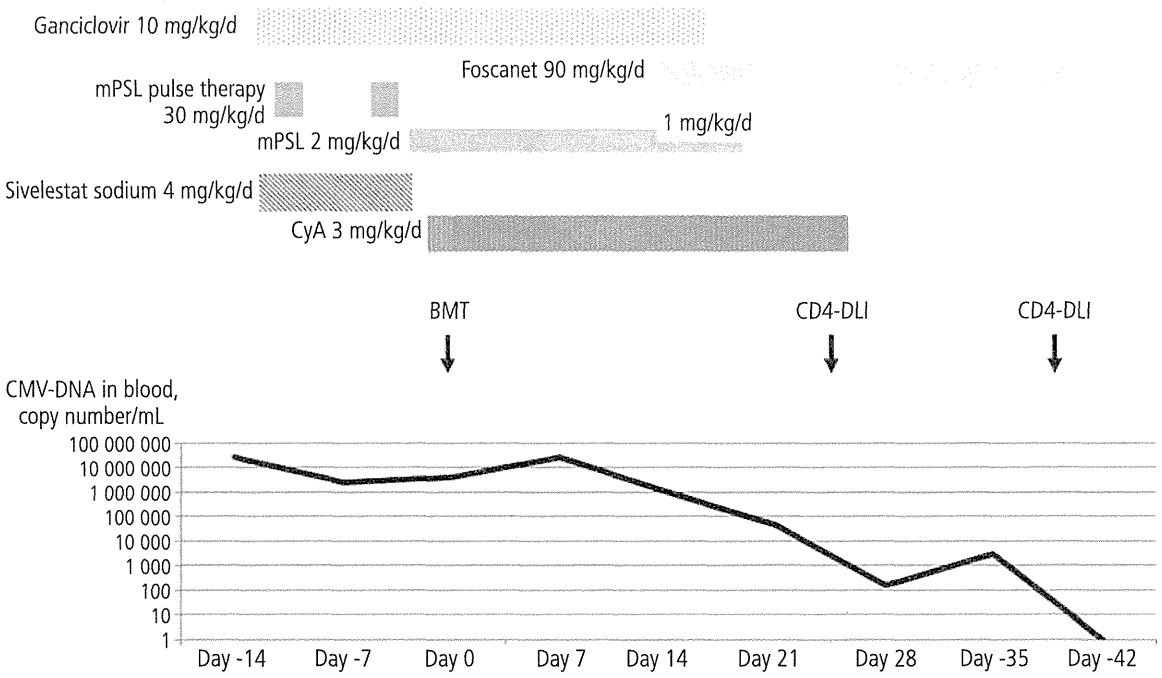

Figure. Clinical course of the patient. The CMV-DNA copy number decreased after BMT followed by CD4-DLI. mPSL indicates methylprednisolone, CyA, ciclosporin A; CMV, cytomegalovirus; BMT, bone marrow transplantation; DLI, donor lymphocyte infusion.

poor outcome. The donor CMV-specific CTLs were transfused to the patient during allogeneic BMT and may have begun to act against CMV infection. CD4-DLI therapy on days 24 and 38 after BMT may also have contributed to the control of CMV infection.

CMV infection is a major complication of allogeneic HSCT. Ganciclovir is an effective antiviral agent, and cidofovir and foscarnet are also effective for CMV infection in ganciclovir-resistant recipients. CMV infection is resistant to these antiviral drugs in certain patients, especially those receiving T-cell–depleted HSCT and those with primary immunodeficiency disease [7]. Treatment with CMV-specific CTLs after HSCT has resulted in cellular immune reconstitution and suppression of viremia [8,9]. The first adoptive immunotherapy involved the use of CMV-specific CTL clones generated by stimulating donor T cells with CMV-infected skin fibroblasts [8]. Dendritic cells and Epstein-Barr virus–transformed lymphoblastoid cell lines have also been used as antigen-presenting cells for expansion of CTLs [9,10]. However, these methods may not be suitable for clinical contexts that require rapid generation of CTLs.

Expansion of autologous T cells from peripheral blood mononuclear cells ex vivo is a feasible and efficacious approach [3] that rapidly enabled complete chimerism to be achieved without graft-versus-host disease in a patient with primary immunodeficiency disease [5]. We performed CD4-DLI for refractory CMV infection in a patient with X-linked SCID. Activated $CD4^+$ T lymphocytes might have a bystander effect for donor-derived CMV-specific CTLs that were transfused during BMT.

In conclusion, we report the case of a patient with X-linked SCID associated with severe CMV infection. The patient recovered from severe CMV infection with CMV-specific CTLs from an HLA-matched sibling donor and adoptive immunotherapy with CD4-DLI.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Molecular and Virological Evidence of Viral Activation From Chromosomally Integrated Human Herpesvirus 6A in a Patient With X-Linked Severe Combined Immunodeficiency

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(See the Editorial Commentary by Flamand on pages 549–51.)

It has been unclear whether chromosomally integrated human herpesvirus 6 (ciHHV-6) can be activated with pathogenic effects on the human body. We present molecular and virological evidence of ciHHV-6A activation in a patient with X-linked severe combined immunodeficiency. These findings have significant implications for the management of patients with ciHHV-6.

Keywords. ciHHV-6; HHV-6; X-SCID; hemophagocytic syndrome; thrombotic microangiopathy.

Human herpesvirus 6 (HHV-6) is a ubiquitous DNA virus that is the causative agent of roseola infantum, and infects individuals by 3 years of age [1]. After primary infection, HHV-6 establishes a latent state in the host. There are 2 distinct species, HHV-6A and HHV-6B. Most HHV-6 infections are caused by HHV-6B, whereas HHV-6A is less common. Chromosomally integrated HHV-6 (ciHHV-6) is the state in which HHV-6 (HHV-6A or HHV-6B) is integrated into the host germline genome, and it is transmitted vertically in a Mendelian manner. Although ciHHV-6 affects about 1% of the general population, it is generally considered to be a nonpathogenic condition. However, it is unclear whether ciHHV-6 can be activated with pathogenic effects on the human body [2].

Severe combined immunodeficiency (SCID) is a group of genetic disorders that result in a combined absence of T- and B-cell immunity. It is characterized by life-threatening infections during the first year of life unless treated, usually with hematopoietic stem cell transplantation (HSCT). X-linked severe combined immunodeficiency (X-SCID) arises from a mutation in the interleukin 2 receptor, gamma (*IL2RG*) gene on the X-chromosome [3]. We encountered a boy with X-SCID in whom ciHHV-6A was activated.

CASE REPORT

A 2-month-old boy was hospitalized for recurrent episodes of fever, cough, diarrhea, and failure to thrive. Upon admission, a viral infection was suspected, and supportive care did not improve his symptoms.

Twenty days after admission, mild pancytopenia (leukocyte count, 1.4×10^9/L; hemoglobin level, 78 g/L; and platelet count, 37×10^9/L) and elevated aminotransferases and ferritin were evident (aspartate aminotransferase, 448 U/L; alanine aminotransferase, 218 U/L; and ferritin, 4325 ng/mL) (Supplementary Figure 1). A bone marrow biopsy showed a hypocellular condition without dysplastic changes, as well as increased activated phagocytes. These results suggested hemophagocytic syndrome (HPS).

An immunological evaluation revealed an absence of T cells and low immunoglobulin levels. Genetic analysis identified a mutation in the *IL2RG* that was consistent with X-SCID. The patient's mother was heterozygous for the same mutation, and there was no such mutation detected in the patient's father.

A comprehensive search for a pathogen identified high levels of HHV-6 DNA (1.2×10^7 copies/μg DNA) in his peripheral blood. Antiviral treatment with ganciclovir or foscarnet did not reduce the viral load, and ciHHV-6 was suspected. We detected high levels of HHV-6 DNA in the patient's fingernails, the father's peripheral blood, and the father's hair follicles (5.9×10^5, 1.0×10^7, 1.2×10^6 copies/μgDNA, respectively).

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Fluorescence in situ hybridization analysis of the patient's fibroblasts and his father's peripheral blood mononuclear cells (PBMCs) confirmed HHV-6 integration at chromosome 22 in both individuals (Figure 1); these results suggested vertical germline transmission.

However, discontinuation of antiviral treatment led to a deterioration of the patient's HPS. Because no other pathogen was detected, activation of HHV-6 was suspected. To confirm this suspicion, we performed 3 assays that could detect viral activation despite the presence of integrated HHV-6 DNA. First, reverse transcription polymerase chain reaction (RT-PCR) was used to detect viral RNA in whole-blood samples. RT-PCR was performed on 2 HHV-6 genes, the late gene U60/66 and the immediate-early (IE) gene IE1, as described previously [5]. We detected viral RNA for both genes (4.6×10^2 copies/μg RNA for U60/66 and 5.2×10^3 copies/μg RNA for IE1). Second, immunostaining was used to detect IE antigens in a bone marrow sample taken at the time of HPS (Figure 2 and Supplementary Figure 2) [6]. Last, HHV-6A was isolated from the patient's PBMCs. It was cultured with cord blood cells and its presence confirmed by immunofluorescent staining with an anti–HHV-6 monoclonal antibody (Figure 3 and Supplementary Figure 3) [1].

Two hypotheses were postulated: Either the patient with ciHHV-6 was infected de novo with HHV-6, or HHV-6 was activated from the ciHHV-6 genome present in this patient. We performed a sequence analysis of the HHV-6 IE1 gene, as IE1 is variable and readily used to distinguish between HHV-6 variants [7]. DNA samples from isolated HHV-6A (described above), the patient's fingernails, his father's hair follicles, and laboratory strains U1102 and Z29 were amplified by PCR and sequenced. Because active HHV-6 is not present in the fingernails or hair follicles, we could amplify the original integrated HHV-6 strain from the genomes in these tissues. To our surprise, the sequences and subsequent phylogenetic analysis revealed that the isolated virus was identical to the original integrated HHV-6A strain present in both the patient and his father. Furthermore, this HHV-6A strain was unique in that it differed from all other HHV-6 strains analyzed (Supplementary Figure 4). These results suggested that the isolated HHV-6A strain originated from the activation of ciHHV-6A. Analysis of 3 other viral genes (gB, U94, and DR) confirmed these results [8, 9].

The resumption of antiviral drug treatment with prednisolone ameliorated the patient's HPS. When he reached age 7 months, the patient underwent HSCT. Antiviral drug treatment was continued during HSCT, and engraftment was achieved 14 days after transplant. After engraftment, thrombotic microangiopathy (TMA) and gastrointestinal bleeding developed. Simultaneously, the patient's HHV-6A DNA and RNA titers increased, and HHV-6A was reisolated. Anticoagulant therapy and a reduction in tacrolimus dosage gradually improved the patient's TMA. With immunological reconstruction, the patient's HHV-6A DNA and RNA titers were successfully reduced and ultimately, no HHV-6A was isolated from subsequent blood samples. The asymptomatic patient was discharged at 12 months.

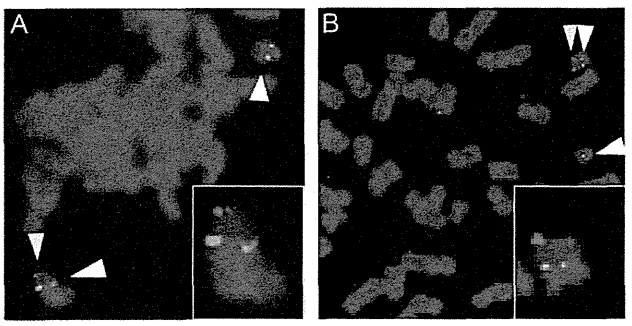

Figure 1. Integration of human herpesvirus type 6 (HHV-6) in chromosome 22 was demonstrated by fluorescence in situ hybridization analysis. Fibroblasts derived from the patient's skin (*A*) and peripheral blood mononuclear cells from the father (*B*) were cohybridized with HHV-6-specific (yellow arrow) and chromosome-22-specific probes (white arrows) [4]. HHV-6 integration in only one of the chromosome 22 alleles was shown in both materials. In sets of *A* and *B* are the enlared images of FISH data positively cohybridized with both probes.

DISCUSSION

Since the discovery of ciHHV-6 in 1993, the question of whether ciHHV-6 can be activated from its integrated state has been perpetually debated [2]. With this case report, we provide the first molecular and virological evidence of viral activation from ciHHV-6A in the human body. This evidence comprises (1) viral RNA and antigens detected in PBMCs and bone marrow, as well as HHV-6A isolated from PBMCs; (2) HHV-6A sequences integrated into the patient's and his father's genomes, which were identical to those of the isolated virus; and (3) antiviral treatment and immunological reconstruction, which were effective in treating this activated ciHHV-6A.

In an effort to understand the biological significance of ciHHV-6, active viral replication from ciHHV-6 has recently been demonstrated in vitro under specific experimental conditions [9–11]. However, only a few studies have suggested ciHHV-6 activation in vivo despite high ciHHV-6 prevalence (approximately 1%) in the general population [12–14]. Activation of ciHHV-6 in vivo has been previously reported in mothers with ciHHV-6 who passed on the infection to infants who did not have inherited ciHHV-6 [8]. Our findings are consistent with these findings, as we clearly demonstrate the activation of HHV-6A in a patient who acquired ciHHV-6 via germline transmission.

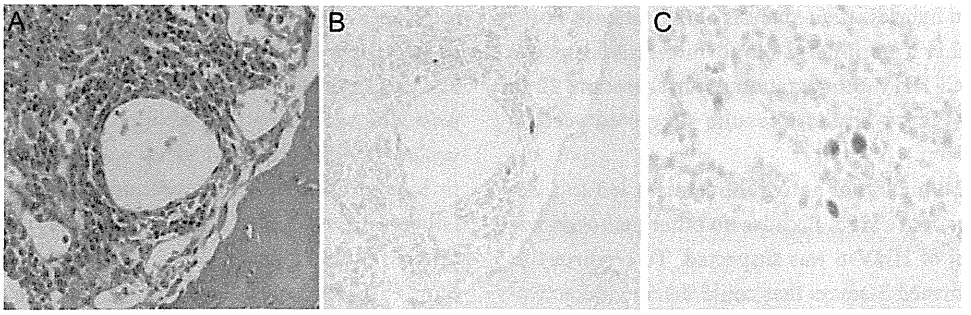

Figure 2. Histology and human herpesvirus type 6 (HHV-6) immunostaining. *A*, Hematoxylin and eosin staining of bone marrow. *B* and *C*, Immunostaining with an anti–HHV-6 antibody.

We speculate that the presence of X-SCID allowed for efficient activation of ciHHV-6A, and this phenomenon was detected with several technical strategies. Similarly, RT-PCR and virus isolation showed conversion from an HHV-6–positive status to a negative status with the patient's immunological recovery. In addition, these techniques were used to test samples taken from the patient's father. We were able to determine that he was indeed the ciHHV-6 carrier, yet he was HHV-6A negative. This suggests that X-SCID influenced the activation of ciHHV-6A. Because X-SCID prevalence is extremely low (about 0.001%), this case provides valuable insight into immunocompromised individuals and HHV-6 infection. However, the mechanism that triggered ciHHV-6A activation and replication in this patient remains to be elucidated. Further studies of patients with ciHHV-6 are required to determine what causes activation of this latent integrated virus.

The association between HHV-6 and HPS has previously been reported [15], and a link between HHV-6 and TMA has also been noted [16]. Therefore, it is possible that ciHHV-6A activation in our patient was associated with HPS and TMA. We noted that active HHV-6A infection coincided with symptom onset and the active infection was controlled with antiviral treatment. This suggests that HHV-6A is pathogenic, yet it remains to be established whether activated HHV-6A enhances underlying pathological conditions, and whether the activation of ciHHV-6A occurs in a similar fashion for all infected individuals.

Latent HHV-6 reactivation occurs in 40%–50% of recipients during HSCT, and our case report is the first to demonstrate that ciHHV-6A activation also occurs during this procedure. It is possible that the presence of X-SCID allowed for viral activation, but further studies are required to validate this hypothesis.

We have described the first case to provide molecular and virological evidence of the activation of chromosomally integrated HHV-6A in the human body. However, our report has limitations. We still do not know how virus production was triggered from a state of ciHHV-6A or how the production of the virus affected the patient's symptoms. Despite these limitations, based on this case, we hypothesize that an immunodeficient phenotype in conjunction with uncontrolled host defense systems allows the activation of ciHHV-6A. We support the recommendation that a screening program to detect ciHHV-6 in

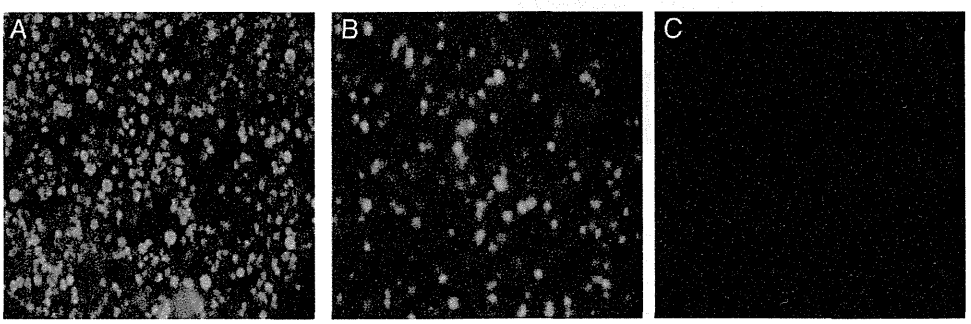

Figure 3. Immunofluorescent staining assay. *A*, Virus isolation confirmed with an anti– human herpesvirus type 6 antibody (gp116/64/54). *B*, U1102 cultured with cord blood cells (positive control). *C*, Cord blood cells alone (negative control).

transplant patients and donors be established, and recommend that ciHHV-6 patients with immunocompromised status such as primary immunodeficiency, human immunodeficiency virus infection, or organ transplantation, be monitored carefully.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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ORIGINAL ARTICLE

Cord blood transplantation is associated with rapid B-cell neogenesis compared with BM transplantation

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Hematopoietic cell transplantation (HCT) is used for treatment of hematopoietic diseases. Assessment of T- and B-cell reconstitution after HCT is crucial because poor immune recovery has a major effect on the clinical course. In this study, we retrospectively analyzed T-cell receptor excision circles (TRECs) as well as signal and coding joint kappa-deleting recombination excision circles (sjKRECs and cjKRECs, respectively) as markers of newly produced lymphocytes in 133 patients (56 primary immunodeficient and 77 malignant cases, median (range): 12 (0–62) years old). We analyzed the kinetics of TREC and KREC recovery and determined the factors that contributed to better immune recovery. KRECs became positive earlier than TRECs and increased thereafter. Younger recipient age had a favorable effect on recovery of sjKRECs and cjKRECs. Compared with BM and peripheral blood, our data suggested that cord blood (CB) provided rapid B-cell recovery. CB also provided better B-cell neogenesis in adult HCT recipients. Chronic GVHD was associated with low TRECs, but not increased sjKRECs/cjKRECs. Finally, positive sjKRECs 1 month after HCT were associated with fewer infectious episodes. Monitoring of TRECs and KRECs may serve as a useful tool for assessment of immune reconstitution post HCT.

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INTRODUCTION

Hematopoietic cell transplantation (HCT) serves as a curative treatment for diseases such as hematopoietic malignancy, congenital BM failure and primary immunodeficiency (PID). Selection of a suitable donor by HLA matching and/or an appropriate conditioning regimen has improved the outcome of HCT for leukemia patients[1] and PID patients.[2,3] Recently, successful outcomes of cord blood transplantation (CBT) and BM transplantation (BMT) have been observed even in HLA-mismatched conditions.[4–8]

Despite these improved outcomes, transplantation-related morbidities such as graft failure, GVHD and infection are still major problems that affect the prognosis and/or quality of life. Infection monitoring after HCT is important for the initiation of preemptive therapy at the appropriate time, while assessment of immune reconstitution is essential because it is considered to be associated with post-transplant infection, relapse of primary disease and OS.[9]

CD4+ T-cell counts, T-cell proliferative capacity, B-cell number and serum IgG have been used as parameters of immune recovery after HCT. Recently, more direct assessment of T- and B-cell neogenesis has become feasible by analyses of T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs), respectively.

DNA fragments between rearranging V, D and J gene segments are deleted as circular excision products during rearrangement of the T-cell receptor gene.[10] These products are called TRECs. Quantitative detection of TRECs enables direct measurement of thymic output. The recovery of TRECs is associated with survival and infection after HCT for treatment of malignancies.[11–13] In a previous study, TREC levels were lower in patients post CBT than in those receiving BMT or PBSC transplantation (PBSCT).[14]

KRECs are formed by Ig kappa-deleting rearrangement during B-cell development. Coding joint KRECs (cjKRECs) serve as an indicator of B-cell numbers,[15] and signal joint KRECs (sjKRECs) are an indicator of B-cell neogenesis. However, the kinetics of KREC recovery post HCT are largely unknown. A correlation between KRECs and survival or infection after HCT has not been reported previously. In addition, whether B-cell recovery as assessed by KRECs is different among graft sources is still unknown.

Here, we investigated the kinetics of TREC and KREC recovery post HCT and factors contributing to better recovery of TREC and KREC levels, mainly focusing on KRECs. We also assessed the association of KRECs with infection after HCT in patients with malignancies or PID.

MATERIALS AND METHODS

Patients

A total of 133 patients who underwent allogeneic HCT from March 1996 to August 2013 were enrolled in this study. The patients were followed up at the Department of Pediatrics or Department of Hematology at Tokyo Medical and Dental University or the Department of Pediatrics of the National Defense Medical College in Japan. The median age at transplantation was 12 years (range, 0–62 years). Table 1 shows the patient characteristics, information on HCT and events associated with transplantation. This study was approved by the ethics committees of

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Table 1. Patient characteristics and clinical course

	PID (56)	*Malignancy (77)*	*All (133)*
Patient characteristics			
Recipient age			
< 18 years	45	44	89
⩾ 18 years	11	33	44
Sex			
Male	42	44	86
Female	14	33	47
Conditioning			
RIC or minimal conditioning	33	10	43
MA	23	66	89
Donor age (BM)			
< 18 years	5	8	13
⩾ 18 years	25	38	63
Cell source			
BM	35	47	82
CB	19	17	36
PB	2	13	15
HLA allele			
⩽ 5/6	20	31	51
6/6	23	33	56
Relation			
Related	13	30	43
Unrelated	43	47	90
Steroid use	28 (52%)	32 (42%)	60 (46%)
ATG use	26 (48%)	5 (6%)	31 (24%)
Clinical course			
Acute GVHD			
Grade 0–2	44	53	97
Grade 3–4	8	10	18
Chronic GVHD	15 (28%)	35 (45%)	50 (38%)
Infection	29 (53%)	48 (62%)	77 (58%)
Bacterial infection	11 (20%)	20 (26%)	31 (23%)
Fungal infection	4 (7%)	6 (8%)	10 (8%)
Viral infection	18 (33%)	35 (45%)	53 (40%)
Relapse	—	30 (39%)	—
Survival	51 (91%)	49 (64%)	100 (75%)

Abbreviations: ATG = antithymocyte globulin; CB = cord blood; MA = myeloablative; PID = primary immunodeficiency; RIC = reduced-intensity conditioning.

Tokyo Medical and Dental University and National Defense Medical College. Informed consent was obtained in accordance with the Declaration of Helsinki.

Measurement of TREC and KREC levels

TREC, sjKREC and cjKREC levels were measured by real-time PCR as described previously[15–19] at 1, 3 and 6 months, and yearly after HCT. RNase P was used as an internal control. Primer and probe sequences are listed in Supplementary Table I. The minimum detectable limit was 10 copies/μg DNA. TRECs or KRECs < 10 copies/μg DNA were defined as negative, and TREC or KREC levels of > 10 copies/μg DNA were defined as positive.

Monitoring of infections

Genomic DNA of eight human herpes virus species, BK virus, JC virus and parvovirus B19 in peripheral blood was measured by multiplex PCR and real-time PCR as described previously.[20] Adenovirus, hepatitis A virus, hepatitis B virus, hepatitis E virus, Norwalk-like virus, Coxsackie virus, ECHO virus, enterovirus, human metapneumovirus and human bocavirus were measured by real-time PCR as described elsewhere.[21] The minimum detectable limit was at least 30 copies/μg DNA.

Definitions

Patients treated with a > 5 Gy single dose of TBI, > 8 Gy fractionated TBI or > 8 mg/kg body weight of BU in addition to other cytoreduction agents were categorized as receiving myeloablative (MA) regimens.[22] HLA typing was performed by genotyping for HLA-A, B and DRB1 loci. GVHD was graded according to standard criteria.[23] We defined the incidence of infection as having symptoms of infection with detectable pathogens and severity ⩾ grade 3 as defined in the Common Terminology Criteria for Adverse Event (CTCAE) version 4.0, National Institutes of Health and National Cancer Institute.

Statistical analysis

Recipient age, recipient sex, disease, conditioning regimen, donor age, cell source, HLA disparity, relationship, acute GVHD, chronic GVHD, and the use of steroids or antithymocyte globulin (ATG) were chosen as clinical parameters. We categorized the diseases of enrolled patients as PID or malignancy. A MA regime was evaluated in comparison with reduced-intensity conditioning regimens and minimal conditioning regimens. HLA-mismatched HCT was compared with 6/6 HLA-matched HCT. Acute GVHD was graded as 0–4 and divided into two groups (0–2 and 3–4). The proportion of surviving patients was estimated by the Kaplan–Meier method and compared using the log-rank test. Factors that were found to be significant ($P < 0.05$) in univariate analysis were included in the multivariate analysis. Multivariate analyses of factors contributing to better TREC/KREC recovery were performed by excluding or including acute and chronic GVHD, steroid use, and ATG use, because these factors are post-HCT events and associated with other factors. Donor age was also excluded because it is restricted for BMT.

RESULTS

Levels of sjKRECs and cjKRECs recover faster than those of TRECs

First, we evaluated the recovery of TREC, sjKREC and cjKREC levels post transplantation.

One month after HCT, TRECs, sjKRECs and cjKRECs were detectable in 17 (17.5%), 34 (35.1%),and 28 (28.9%) of 97 patients, respectively. The median copy number was low (< 10 copies/μg DNA) in all assays (Figures 1a and b).

Eighty-two patients were examined 3 months after HCT. TRECs were positive in 15 (18.3%) patients, whereas sjKRECs and cjKRECs were positive in 57 (69.5%) and 59 (72.0%) patients, respectively (Figures 1c and d).

TRECs became positive in 41.3% of patients at 6 months and in 66.7% of patients 1 year post HCT. SjKRECs and cjKRECs were positive in 77.8% of patients at 6 months and in > 90% of patients at 1 year post HCT. The median level of TRECs was < 10 copies/μg DNA at 6 months and reached up to 1270 copies/μg DNA at 1 year. Interestingly, sjKRECs continued to increase for at least 1 year, while cjKRECs peaked at 6 months, and then started to decline (Figures 1e–h).

The recovery of sjKREC and cjKREC levels correlated as shown in Figures 1b, d, f, and h. This finding indicates that B-cell maturation is intact once B-cell engraftment is achieved. On the other hand, a considerable number of patients exhibited B-cell neogenesis in the absence of T-cell neogenesis, especially at the early stage post HCT (Figures 1a, c, and e).

We examined the trend of TRECs and KRECs in individual patients, of whom 71% had positive sjKRECs at 1 month and showed increased sjKRECs at 3 months. Similarly, the levels of sjKRECs detectable at 1 month increased at 6 months in 80% of the patients (Figure 2b). On the other hand, positive TRECs at 1 month did not indicate further T-cell recovery at a later period. When we examined patients with positive TRECs at 3 months, 10 of the 11 patients had increased TREC levels at 6 months, suggesting that positive TRECs at 3 months may serve as a predictor of T-cell reconstitution after 6 months (Figure 2a).

Longitudinal analysis showed that the recovery course of TRECs from 1 month to 15 years post HCT is at least not inferior to CBT when compared with that of BMT and PBSCT. Compared to BMT,

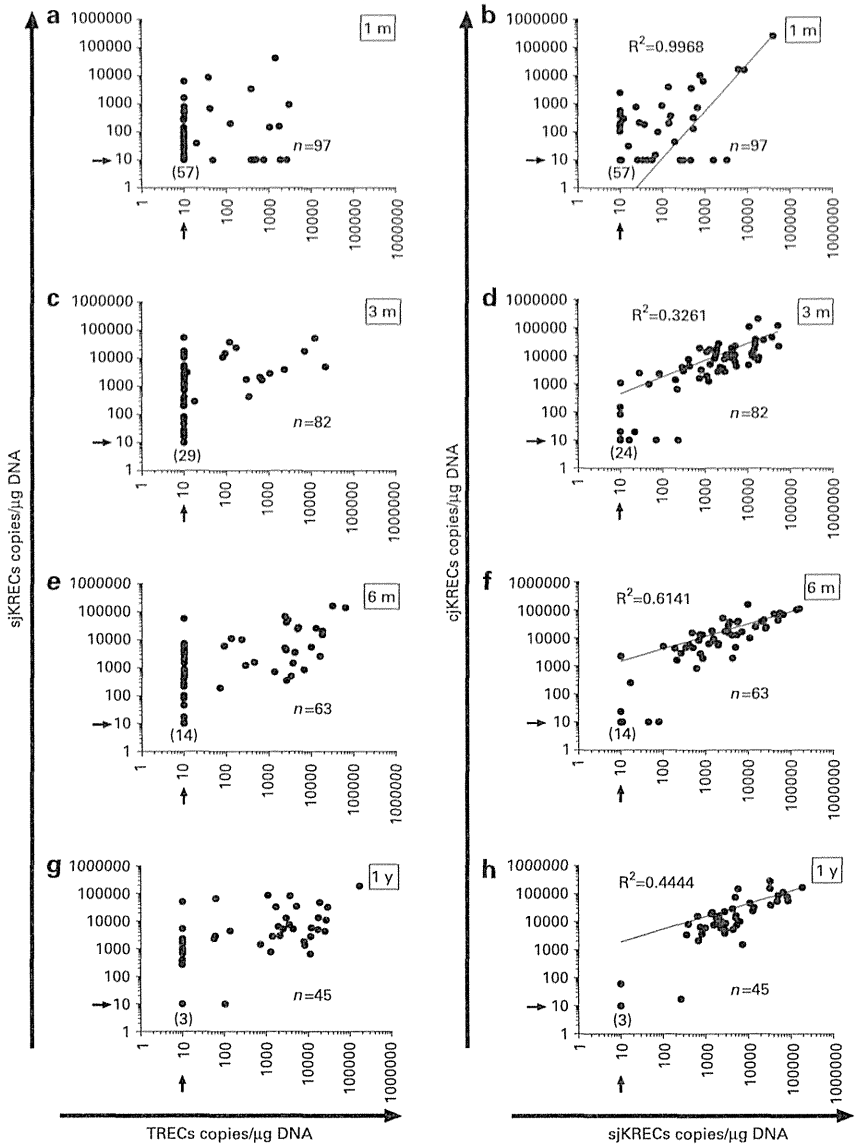

Figure 1. Recovery of TREC and KREC levels. Recovery of the levels of TRECs and sjKRECs, and sjKRECs and cjKRECs at 1 month (**a**, **b**), 3 months (**c**, **d**), 6 months (**e**, **f**) and 1 year (**g**, **h**) after HCT. Arrows show the detectable limit of the real-time PCR (10 copies/μg DNA). Values under the limit are considered 'negative'. Numbers in parentheses indicate the number of subjects who show a negative value (< 10 copies/μg DNA) for the indicated products.

KREC levels recovered more rapidly after CBT (Supplementary Figures 1 and 2). Final sjKREC/cjKREC levels reached the levels of the age-matched control when KRECs were fully recovered (data not shown).

Younger recipient age and CB favor increased levels of sjKRECs and cjKRECs

Next, we evaluated the factors that contributed to the levels of TRECs, sjKRECs and cjKRECs by regression analysis, including the factors listed in Materials and Methods (Supplementary Table II). A younger recipient and donor age was defined as < 18 years old.

In univariate analysis, a younger recipient age was a favorable factor for increased levels of TRECs, sjKRECs or cjKRECs post HCT. In fact, only the cjKREC levels of older recipients became close to those of younger recipients at 2 years after HCT (Figure 3). In BMT recipients, a younger donor age was a favorable factor for increased levels of TRECs, sjKRECs and cjKRECs (Supplementary Table II and Supplementary Figure 3). Compared with BM or PB, the use of CB was a favorable factor for increased levels of sjKRECs and cjKRECs after HCT (Figure 4 and Supplementary Table II).

A MA regime, PID, no or mild acute GVHD (grade 0–2), no chronic GVHD, no use of steroids, and no use of ATG were also favorable factors for increased levels of TRECs, sjKRECs or cjKRECs at various time points after HCT (Supplementary Table II).

On the basis of the results obtained from the univariate analysis, the following factors were used in multivariate analysis: recipient age, disease, conditioning regimen, cell source and relationship. Our results concerning TRECs largely reconfirmed previous reports,[13,24] indicating that a younger recipient age, no ATG use and a MA regime are associated with better TREC recovery. When focusing on B-cell recovery, we found that a younger recipient age was a favorable factor for increased levels of sjKRECs at 6 months to 2 years and cjKRECs at 6 months to 1 year after HCT. In addition, compared with BMT, CBT favored increased levels of sjKRECs at 1, 3 and 48 months. A MA regime was a favorable factor for increased levels of sjKRECs at 3 to 6 months (Table 2).

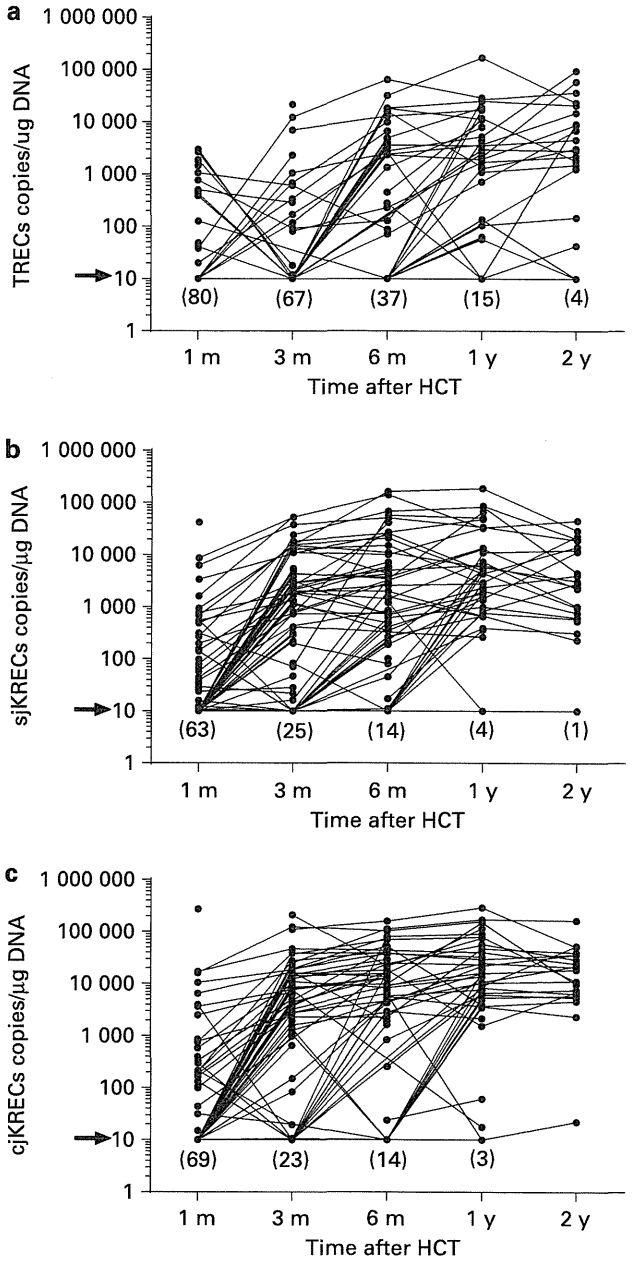

Figure 2. Levels of TRECs and KRECs after HCT. The levels of TRECs (**a**), sjKRECs (**b**) and cjKRECs (**c**) after HCT. The arrows show the detectable limit of the real-time PCR (10 copies/μg DNA). Values under this limit are considered 'negative'. Numbers in parentheses indicate the number of subjects who show a negative value (< 10 copies/μg DNA) for the indicated products.

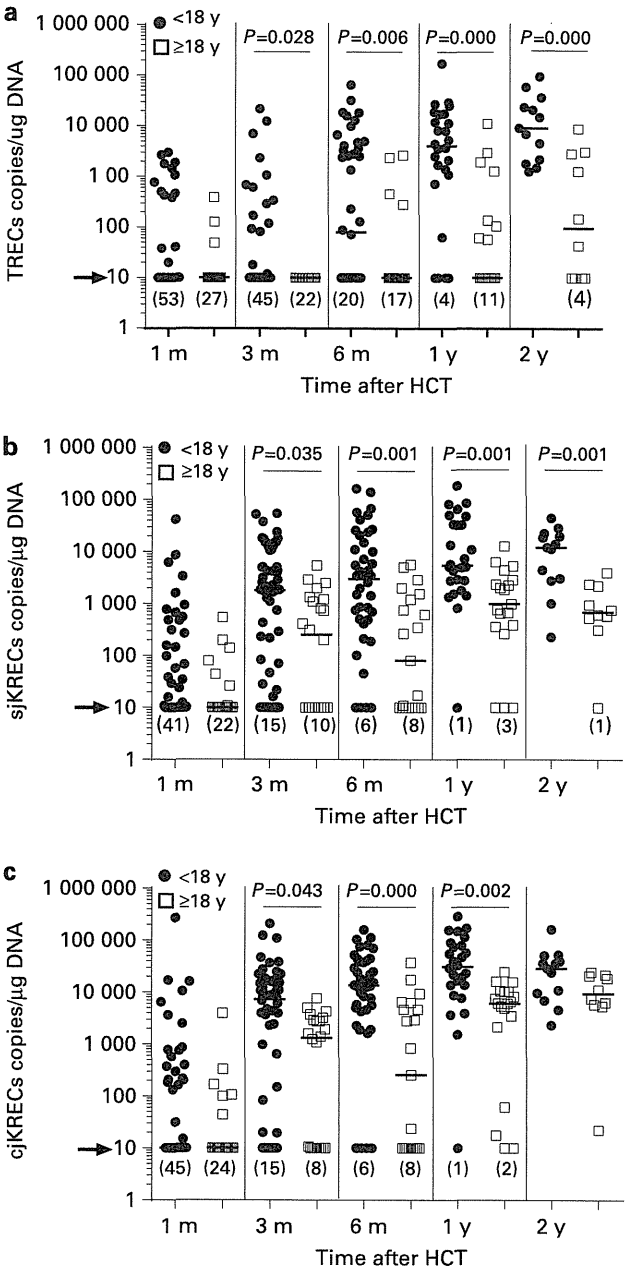

Figure 3. Recipient age and the levels of TRECs and KRECs. Recipient age and the levels of TRECs (**a**), sjKRECs (**b**) and cjKRECs (**c**). Closed circles indicate < 18 years old, and open squares indicate ⩾ 18 years old. Arrows show the detectable limit of the real-time PCR (10 copies/μg DNA). Values under this limit are considered 'negative'. Numbers in parentheses indicate the number of subjects who show a negative value (< 10 copies/μg DNA) for the indicated products.

By including acute GVHD, steroid use and ATG use in the analysis, grade 0–2 acute GVHD, no steroid use and no ATG use were identified as factors favoring better KREC recovery at various time points (Supplementary Table III). The analysis further including chronic GVHD suggested that the condition does not affect B-cell neoproduction (Supplementary Table IV).

We then performed multivariate analysis of a group of patients with malignancy. Compared with BM recipients, the results showed that sjKRECs and cjKRECs were more frequently detectable in CB recipients at 3 months (Supplementary Table V). Compared with BM recipients, in adult patients of ⩾18 years of age ($n=44$), the use of CB was a favorable factor for increased levels of sjKRECs at 1 month (Supplementary Table VI). These data show that CB use contributes to early recovery of neogenesis. In contrast, we observed no significant difference of T-cell recovery in adult patients when CB use was compared with BM at any time point after HCT (Supplementary Figure 4).

Positivity for sjKRECs 1 month after HCT is associated with decreased infectious episodes

We next investigated whether the levels of TRECs, sjKRECs or cjKRECs were associated with the occurrence of infections. We found that positive sjKRECs or TRECs 1 month after HCT correlated

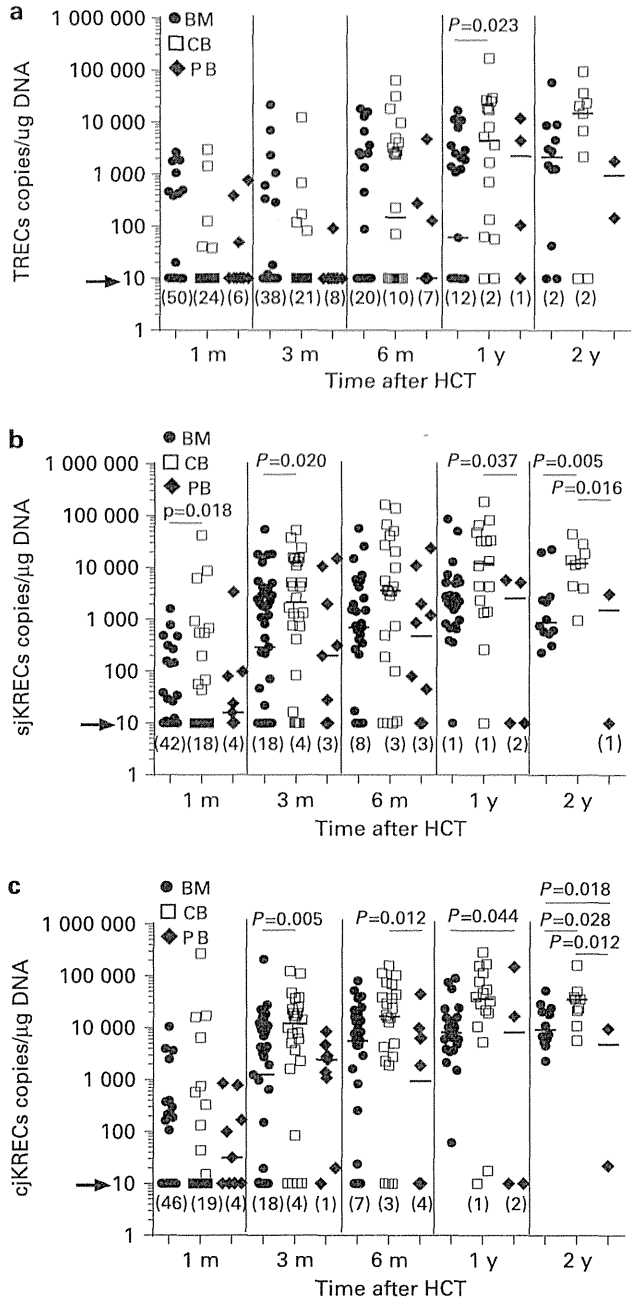

Figure 4. Cell source and the levels of TRECs and KRECs. Cell source and the levels of TRECs (**a**), sjKRECs (**b**) and cjKRECs (**c**). Closed circles indicate BM, open squares indicate cord blood and closed diamonds indicate peripheral blood. Arrows show the detectable limit of the real-time PCR (10 copies/μg DNA). Values under the limit are considered 'negative'. Numbers in parentheses indicate the number of subjects who show a negative value (< 10 copies/μg DNA) for the indicated products.

with decreased infectious episodes (Figure 5). Sixteen out of 34 patients who were positive for sjKRECs suffered from infections, whereas 43 of 63 patients who were negative for sjKRECs acquired infections (Figure 5b, $P=0.033$).

We also examined the association between each index and the incidence of infectious episodes caused by bacteria, fungi or viruses. Although there was a tendency toward less bacterial infections in sjKREC- or cjKREC-positive groups, we found no statistical significance. Cumulative incidence of each infection did

Table 2. Multivariate analysis of factors that contributed to the levels of TRECs, sjKRECs and cjKRECs

Factors	*1 month*		*3 months*		*6 months*		*1 year*		*2 years*	
	β (95% CI)	*P-value*	*β (95% CI)*	*P-value*	*β (95% CI)*	*P-value*	*β (95% CI)*	*P-value*	*β (95% CI)*	*P-value*
TRECs										
Younger recipient age	0.084 (−0.213 to 0.449)	0.481	0.140 (−0.161 to 0.633)	0.241	0.231 (−0.133 to 1.400)	0.104	**0.379 (0.263 to 1.829)**	**0.010**[a]	**0.629 (0.662 to 2.511)**	**0.002**[a]
PID	0.217 (−0.077 to 0.665)	0.119	0.120 (−0.221 to 0.583)	0.373	0.126 (−0.505 to 1.158)	0.435	**0.348 (0.155 to 1.769)**	**0.021**[a]	0.355 (−0.254 to 2.118)	0.115
MA	**0.354 (0.137 to 0.853)**	**0.007**[a]	0.166 (−0.134 to 0.648)	0.194	0.049 (−0.635 to 0.893)	0.737	0.154 (−0.338 to 1.180)	0.269	−0.117 (−1.217 to 0.632)	0.512
BM (compared with CB)	0.088 (−0.194 to 0.428)	0.456	−0.071 (−0.488 to 0.275)	0.580	−0.183 (−1.241 to 0.296)	0.223	−0.256 (−1.514 to 0.111)	0.088	−0.018 (−0.925 to 0.837)	0.916
PB (compared with CB)	0.223 (−0.114 to 1.113)	0.109	−0.254 (−1.338 to 0.130)	0.105	−0.286 (−2.415 to 0.391)	0.154	−0.106 (−2.046 to 1.030)	0.508	0.033 (−1.742 to 2.032)	0.873
Relation	−0.075 (−0.491 to 0.272)	0.571	**0.344 (0.115 to 1.000)**	**0.014**[a]	0.220 (−0.345 to 1.568)	0.206	0.195 (−0.312 to 1.487)	0.194	0.116 (−0.735 to 1.367)	0.532
sjKRECs										
Younger recipient age	0.070 (−0.282 to 0.552)	0.553	0.072 (−0.427 to 0.841)	0.517	**0.339 (0.241 to 1.618)**	**0.009**[a]	**0.328 (0.060 to 1.292)**	**0.032**[a]	**0.414 (0.071 to 1.333)**	**0.031**[a]
PID	0.160 (−0.187 to 0.715)	0.248	0.178 (−0.186 to 1.098)	0.161	−0.053 (−0.883 to 0.610)	0.716	0.236 (−0.147 to 1.123)	0.128	0.282 (−0.310 to 1.308)	0.210
MA	0.167 (−0.151 to 0.719)	0.198	**0.263 (0.069 to 1.318)**	**0.030**[a]	**0.299 (0.098 to 1.471)**	**0.026**[a]	0.107 (−0.378 to 0.817)	0.462	0.069 (−0.514 to 0.747)	0.701
BM (compared with CB)	**−0.258 (−0.796 to −0.041)**	**0.030**[a]	**−0.372 (−1.566 to −0.347)**	**0.003**[a]	−0.230 (−1.284 to 0.095)	0.090	−0.181 (−1.010 to 0.268)	0.247	**−0.370 (−1.224 to −0.022)**	**0.043**[b]
PB (compared with CB)	−0.098 (−1.012 to 0.479)	0.479	**−0.407 (−2.824 to −0.480)**	**0.006**[b]	−0.349 (−2.495 to 0.025)	0.055	**−0.447 (−2.809 to 0.389)**	**0.011**[b]	−0.420 (−2.540 to 0.034)	0.056
Relation	0.149 (−0.200 to 0.727)	0.262	**0.394 (0.381 to 1.793)**	**0.003**[a]	0.194 (−0.320 to 1.398)	0.214	0.218 (−0.219 to 1.197)	0.170	−0.019 (−0.751 to 0.682)	0.920
cjKRECs										
Younger recipient age	0.017 (−0.450 to 0.519)	0.887	0.085 (−0.429 to 0.967)	0.445	**0.352 (0.309 to 1.822)**	**0.007**[a]	**0.383 (0.144 to 1.563)**	**0.020**[a]	0.171 (−0.375 to 0.869)	0.412
PID	**0.287 (0.024 to 1.112)**	**0.041**[a]	0.148 (−0.285 to 1.128)	0.239	−0.052 (−0.969 to 0.671)	0.717	0.145 (−0.407 to 1.055)	0.376	0.305 (−0.339 to 1.257)	0.240
MA	0.216 (−0.083 to 0.966)	0.098	**0.281 (0.136 to 1.510)**	**0.020**[a]	0.257 (−0.007 to 1.500)	0.052	0.015 (−0.654 to 0.721)	0.921	0.071 (−0.520 to 0.724)	0.733
BM (compared with CB)	−0.164 (−0.775 to 0.135)	0.166	**−0.427 (−1.890 to −0.548)**	**0.001**[a]	−0.189 (−1.297 to 0.219)	0.160	−0.033 (−0.810 to 0.662)	0.840	−0.261 (−0.967 to 0.218)	0.199
PB (compared with CB)	−0.026 (−0.985 to 0.812)	0.849	**−0.329 (−2.770 to −0.190)**	**0.025**[b]	**−0.422 (−3.035 to −0.267)**	**0.020**[b]	−0.346 (−2.730 to 0.056)	0.059	**−0.580 (−2.747 to −0.208)**	**0.025**[b]
Relation	0.158 (−0.225 to 0.893)	0.238	**0.336 (0.253 to 1.808)**	**0.010**[a]	0.160 (−0.454 to 1.433)	0.303	0.154 (−0.441 to 1.188)	0.359	0.001 (−0.706 to 0.708)	0.998

Abbreviations: CB = cord blood; cjKREC = coding joint kappa-deleting recombination excision circle; MA = myeloablative; PB = peripheral blood; PID = primary immunodeficiency; sjKREC = signal joint kappa-deleting recombination excision circle; TREC = T-cell receptor excision circle. Bold letters indicate significant factors. [a]Significant favorable factors that contributed to the levels of TRECs, sjKRECs and cjKRECs. [b]Significant unfavorable factors that contributed to the levels of TRECs, sjKRECs and cjKRECs.