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Reconstitution of Protective Immune Responses against Cytomegalovirus and Varicella Zoster Virus Does Not Require Disease Development in Pediatric Recipients of Umbilical Cord Blood Transplantation

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CMV and varicella zoster virus (VZV) are significant causes of morbidity and mortality following umbilical cord blood transplantation (UCBT). However, the kinetics of reconstitution and protective potential of antiviral cell-mediated immune responses following UCBT remain poorly characterized. In this study, the reconstitution of CMV- and VZV-specific T cell responses was assessed using IFN-γ ELISPOT in 28 children who underwent UCBT to treat hematological or inherited disorders. Barely detectable in the first 3 mo posttransplantation, CMV- and VZV-specific T cell responses were observed in 30.4% and 40.3% of study subjects after 36 mo of follow-up. Four of five CMV-seropositive subjects developed detectable levels of circulating CMV DNA (DNAemia), and 5 of 17 VZV-seropositive patients experienced herpetic zoster during the posttransplant period. Four CMV-seronegative subjects developed IFN-γ responses against CMV, and four subjects developed a VZV-specific IFN-γ response without clinical signs of infection. No CMV- or VZV-related events were observed in study subjects following the development of CMV- or VZV-specific responses > 150 spot-forming units/10⁶ PBMCs, consistent with T cell-mediated protection. Finally, famciclovir prophylaxis did not strictly prevent the reconstitution of the VZV-specific T cell repertoire, because the frequency of T cells producing IFN-γ in response to VZV Ags reached levels consistent with protection in two nonzoster subjects. Monitoring of CMV- and VZV-specific cell-mediated immunity could inform immunocompetence and guide the initiation and cessation of antitherpetic prophylaxis in UCBT recipients. The Journal of Immunology, 2012, 189: 000–000.

A s an alternative to unrelated bone marrow (BM) transplantation (BMT), umbilical cord blood (UCB) transplantation (UCBT) has been used for >2 decades to treat pediatric patients suffering from various hematological disorders. T lymphocytes contained in UCB exhibit similar subset distribution as those found in adult peripheral blood. However, they display a mostly naive phenotype as well as reduced alloreactivity and heightened susceptibility to apoptosis (reviewed in Ref. 1). Furthermore, the T lymphocyte compartments found in UCB and BM allograft inculums differ extensively from one another in quantitative and qualitative terms. The numbers of CD3⁺ cells are approximately six times lower in a typical UCBT graft compared with a BMT inoculum, and the CD4⁺/CD8⁺ T cell ratio is significantly higher (3.0 versus 1.4) (2). In addition, UCB is a rich source of CD4⁺FOXP3⁺ regulatory T cells that possess potent suppressor function in MLR (3). That likely explains the specific pattern of clinical outcomes associated with UCBT in comparison with BMT. Major advantages of UCBT include a low incidence of graft-versus-host disease (GVHD) (4) and an efficient graft-versus-leukemia effect (5). Main disadvantages include delayed en-
graft, late reconstitution of the CD8+ T cell subset, and a higher incidence of opportunistic infections in the first 3–6 mo posttransplantation that results in higher morbidity and mortality relative to BMT during this period (6–8). In particular, CMV and varicella zoster virus (VZV) are important causes of opportunistic infections, and infections with these pathogens are observed more frequently in UCBT recipients than in BMT recipients (9, 10).

CMV is a lymphotropic herpesvirus that establishes latency and persists in the host following primary infection. CMV infection is largely asymptomatic in immunocompetent hosts. However, in immunodeficient subjects, it is associated with life-threatening complications, including pneumonia, gastroenteritis, and, less commonly, retinitis, hepatitis, and encephalitis (11). Between 50 and 100% of patients that are CMV seropositive prior to transplantation will experience clinical symptoms of CMV infection and/or detectable viral replication by day 100 post-UCBT (12–14). Ganciclovir prophylaxis does not fully suppress CMV reactivation and is associated with myelosuppression that limits its use in children (7, 15–17). Pre-emptive treatment based on monitoring using weekly pp65-specific PCR assays during the first 6 mo following transplantation decreases the incidence of CMV disease to 5–20% at day 100 (14, 18, 19). However, late-onset (>6 mo) CMV disease can still occur and is associated with high mortality, particularly in CMV-seropositive recipients (20–22). Moreover, the high sensitivity of PCR detection may lead to overtreatment of patients who would not have otherwise progressed to overt CMV disease (23). Recent tetramer-based studies showed that rapid recovery of CMV-specific CD8+ T lymphocytes was associated with a reduced incidence of CMV-related complications in allogeneic hematopoietic stem cell transplantation (HSCT) recipients (24, 25). Production of IFN-γ by CMV-specific CD4+ and CD8+ T cells was closely associated with control of CMV antigenemia and circulating levels of CMV DNA (DNAemia) following allogeneic HSCT in some studies (26, 27) but not in others (28, 29), all of which were studies in which only a few pediatric UCBT recipients were enrolled.

VZV is a α-herpesvirus that preferentially infects epithelial and neural cells. Primary infection with VZV causes varicella, whereas its reactivation causes herpes zoster. VZV infection can lead to the development of threatening complications in immunocompromised hosts, including visceral dissemination to the lungs, liver, and CNS (30–32). VZV reactivation is common following HSCT, with 13–55% of patients developing VZV-associated complications in the first year (31). VZV reactivation is more frequent (63%) and more severe, and it occurs later following UCBT compared with BMT (9). For this reason, long-term prophylaxis with acyclovir or valacyclovir was recommended to prevent VZV-related complications in HSCT recipients (33). Famiciclovir is used for the same indication at Centre Hospitalier Universitaire Sainte-Justine. However, there are concerns about whether this prophylactic strategy may also lead to overtreatment and/or development of antiviral drug resistance. In addition, because long-term protection from CMV and VZV infections critically depends on the reconstitution of a functional antiviral T cell repertoire (34–38), concerns were raised that effective antiviral prophylaxis might impair virus-specific immune reconstitution (39, 40). Indeed, using IFN-γ production as a marker of T cell function, Distler et al. (41) reported that VZV-specific T cell-mediated immunity was recovered efficiently in T cell-depleted allogeneic BMT recipients who experienced herpes zoster but not in nonzoster patients, suggesting that VZV replication was required for priming/restoration of efficient antiviral T cell responses.

With this in mind, the objectives of the current study were 4-fold: to define and compare the kinetics of reconstitution of CMV-specific and VZV-specific T cell-mediated immune responses following UCBT; to determine whether CMV- or VZV-associated complications are required for reconstitution of cognate cell-mediated immune responses; to determine whether these responses protect the graft recipient against CMV- and VZV-associated complications; and to examine whether the development of these responses is influenced by antiviral prophylaxis. To do this, the reconstitution of CMV- and VZV-specific T cell responses was monitored using ELISPOT between day 1 and 36 mo posttransplantation in a group of 28 children who underwent UCBT for the treatment of hematological malignancies (n = 24) or other hematological or metabolic disorders (n = 4). Results indicate that CMV- and VZV-specific IFN-γ responses are restored with similar kinetics, that an important proportion of subjects developed CMV- and VZV-specific IFN-γ responses without manifesting symptoms of the diseases, that emergence of robust CMV- or VZV-specific cell-mediated immune responses was associated with absence of subsequent CMV- and VZV-associated clinical manifestations, and that famciclovir prophylaxis did not impact the reconstitution of VZV-specific cell-mediated immunity.

Materials and Methods

Patient characteristics

This research protocol was approved by the Institutional Ethics Review Board of Centre Hospitalier Universitaire Sainte-Justine, where the study was conducted. Twenty-eight subjects who underwent either single unrelated UCBT (n = 26) or double unrelated UCBT (n = 2) were enrolled between October 2004 and June 2010. Patients P1–P26 were the subject of a recent report focused on reconstitution of T cell responses directed against the Melan-A tumor Ag following UCBT (42). Median age at transplantation was 65 mo (range: 4–211 mo). Venous blood samples (2–10 ml) were obtained from transplant recipients at 1, 2, 3, 6, 9, 12, 18, 24, and 36 mo posttransplantation. PBMC were isolated on Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) and cryopreserved in 90% v/v FBS (Invirotech, Burlington, ON, Canada) supplemented with 10% v/v DMSO. Clinical and sociodemographic characteristics of study subjects are summarized in Table I. Detailed transplant procedures were described elsewhere (43). Briefly, UCB units, procured from national and international cord blood banks, were required to show a 4/6 or greater allele-level match (HLA-A, HLA-B, HLA-DRB1) with the patient and with each other in the case of double UCBT. The conditioning regimen included total-body irradiation or busulfan. Graft inoculums (i.e., the cells that are actually transplanted into the graft recipient) were not in vitro T cell depleted. GvHD prophylaxis consisted of cyclosporine A and corticosteroids for 1 mo, as well as sorotherapy with 2 mg/kg anti-thymocyte globulin (Thymoglobulin; Sangstat, Mississauga, ON, Canada) on days +2 and +2. Subjects were also treated with filgrastim (Neupogen; Amgen, Mississauga, ON, Canada) until 5 d postneutrophil recovery. Intravenous Ig (500 mg/kg; multiple suppliers) were administered weekly to all subjects from transplant to day +100 and then every month for 6 mo. Subjects with pretransplant positive serology for HSV were given acyclovir prophylaxis (250 mg/m²/12 h) from days –1 to +21.

Monitoring, antiviral prophylaxis, and treatment of CMV and VZV infections

Pretransplant CMV serological positivity in the graft recipients (IgG Abs to CMV late Ags) was confirmed using ELISA. In subjects enrolled before September 2005, CMV reactivation was monitored by detection of CMV pp67 mRNA in whole blood using a semiquantitative nucleic acid sequence-based amplification (NASBA) method (44). An in-house real-time PCR method based on detection of pp65 was then implemented starting in 2005 (primer sequences are available upon request). Monitoring was performed on a weekly basis until 6 mo posttransplantation or longer if immunosuppression persisted. Ganciclovir treatment (5 mg/kg/12 h) was initiated upon positive NASBA or detection of >3000 CMV DNA copies/ml plasma and discontinued upon disappearance of viremia. The term “CMV DNAemia” is used in the text to denote detection of CMV using either NASBA or PCR-based methods. VZV serological status prior to and posttransplantation was determined using ELISA (Enzygnost; Dade Behring Marburg, Marburg, Germany; Euroimmun, Lübeck, Germany). VZV disease was suspected in the presence of typical cutaneous vesicular lesions and confirmed by virus isola-
tion using standard culture or Ag detection by direct immunofluorescence. Anti-VZV Ig (VZIG; Cangene, Winnipeg, AB, Canada) were administered to one subject (P5) at 18 mo posttransplant following possible VZV exposure. Since 2007, VZV-seropositive subjects were treated with famciclovir prophylaxis for the first year following UCBT (no treatment in children <2 y of age; 125 mg, twice a day, in children aged 2–5 y; 250 mg, twice a day, in children aged 6–11 y; 500 mg, twice a day, in children aged >12 y). When zoster occurred after 12 mo posttransplantation, subjects were treated with i.v. acyclovir for 3 d (500 mg/m², three times/d, followed by 10 d of treatment with famciclovir (250 mg, three times/d in children weighing between 20 and 40 kg; 500 mg, three times/d in children >40 kg).

**Immunophenotyping**

Absolute CD4⁺ and CD8⁺ T lymphocyte counts were determined by flow cytometry. Phenotypic analysis of CD8⁺ T cells was performed using cryopreserved PBMC, whereas CD4⁺ T cells were phenotyped from fresh whole blood. Except where mentioned, all mAbs were purchased from BD Biosciences (San Diego, CA). The following mAbs were used: PE- or allophycocyanin-conjugated anti-CD8 (RPA-T8), PE-Cy7–conjugated anti-CD45RA (HI100), or allophycocyanin-conjugated anti-CD4 (SK3), PE-conjugated anti-CD45RA, and FITC-conjugated anti-CD45RA (ALB11; Beckman Coulter, Indianapolis, IN), and FITC-conjugated anti-CD31 (PECAM-1, clone WM59) (46). mAbs for cell surface staining were PE-conjugated anti-CD4 (SK3), PE-conjugated anti-CD45RA (HI100) (45), or allophycocyanin-conjugated anti-CD4 (SK3), PE-conjugated anti-CD45RA (ALB11; Beckman Coulter, Indianapolis, IN), and FITC-conjugated anti-CD31 (PECAM-1, clone WM59) (46). mAbs for cell surface staining were added, and cells were incubated for 30 min at room temperature for CD8⁺/CD31 (PECAM-1, clone WM59) (46). PE-conjugated anti-CD4 (SK3) (45), or allophycocyanin-conjugated anti-CD4 (SK3), PE-conjugated anti-CD45RA (ALB11; Beckman Coulter, Indianapolis, IN), and FITC-conjugated anti-CD31 (PECAM-1, clone WM59) (46). mAbs for cell surface staining were added, and cells were incubated for 30 min at room temperature for CD8⁺ T cells. In the case of CD4⁺ T cells, incubation was on ice for 20 min, after which erythrocytes were lysed using 10% v/v BD FACS Lysing Solution. The human CMV pp65 (NLVPMV ATV) (table I). Twenty-six were transplanted with a single UCB unit, and two were transplanted with double UCB units. Nine subjects (32.1%) died during the 36-mo follow-up. Five subjects (17.9%) had detectable anti-CMV Ab titers (CMV seropositive) prior to

ELISPOT

Frozen PBMC were thawed in the presence of Benzonase Nuclease (Novagen, Madison, WI) and suspended in RPMI 1640 supplemented with 20% v/v FBS and 50 μg/ml gentamicin (Invitrogen). Cells were incubated overnight at a concentration of 2 × 10⁶ cells/ml at 37°C under a 5% CO₂ atmosphere. Assays were performed using 96-well Multiscreen IP plates (Millipore, Bedford, MA) coated overnight at 4°C with purified mouse anti-human IFN-γ capture Ab (NIB4; BD Biosciences) and alkaline phosphatase-conjugated streptavidin (Bio-Rad, Hercules, CA). Plates were washed and incubated with BCIP/NBT-plus substrate solution (Bio-Rad). All assays were performed in duplicates. Spots were enumerated using a CTL ImmunoSpot S4 ultraviolet Analyzer (Cellular Technology, Shaker Heights, OH). Background was subtracted, and IFN-γ production was expressed as the number of spot-forming units (SFU)/10⁶ PBMC. Samples were considered positive if the number of SFU was >50/10⁶ PBMC and 2 SD above the negative control. A value of 400 SFU/well was used when spots were too numerous to be counted.

**Statistical analysis**

The Wilcoxon rank-sum test and the Kruskal–Wallis test with the Dunn multiple comparison test were used to assess differences between continuous variables, whereas the χ² test was used for categorical measurements. Correlations between variables were tested using the Spearman rank-correlation test. Reconstitution of CMV- and VZV-specific cell-mediated immune responses was represented using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Statistical analysis was performed using GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

**Results**

**Characteristics of study subjects**

A total of 28 children with hematological disorders was enrolled in this study, most of whom were diagnosed with malignant diseases (Table I). Twenty-six were transplanted with a single UCB unit, and two were transplanted with double UCB units. Nine subjects (32.1%) died during the 36-mo follow-up. Five subjects (17.9%) had detectable anti-CMV Ab titers (CMV seropositive) prior to

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**Table I. Clinical characteristics of study subjects**

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<td>Outcome</td>
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<td>Graft failure</td>
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FHHL, familial hemophagocytic lymphohistiocytosis; WAS, Wiskott–Aldrich syndrome.

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transplantation, and 17 (60.7%) had measurable anti-VZV Ab titers (VZV seropositive) prior to transplantation. In all, 14 subjects (50.0%) developed at least one complication related to infection with herpesviruses, including 11 (39.3%) during the first 100 d posttransplantation. These early complications involved HSV-1 \((n = 4)\), CMV \((n = 4)\), EBV \((n = 2)\), VZV \((n = 2)\), and human herpes virus 6 \((n = 1)\). Two recurrences of zoster and three additional VZV reactivations occurred 1–2 y posttransplantation (Table I).

**Reconstitution of CD4⁺ and CD8⁺ T lymphocyte subsets**

As expected, absolute CD4⁺ and CD8⁺ T cell counts were extremely low in the first 3 mo posttransplantation. Although CD4⁺ T cell counts doubled between 2 and 3 mo \((median, 25 and 52 cells/mm³ at 2 and 3 mo posttransplantation, respectively)\), CD8⁺ T cell counts remained stable \((median, 16.3 and 18 cells/mm³, respectively)\). Median CD4⁺ and CD8⁺ T cell counts remained below normal at 6 mo posttransplantation, although some subjects had already attained normal T lymphocyte counts \((Fig. 1A)\) \((51, 52)\). The vast majority of subjects recovered levels of T cells consistent with normal values by 12 mo posttransplantation \((median, 928 and 435 cells/mm³ for CD4⁺ and CD8⁺ T lymphocytes, respectively)\) \((Fig. 1A)\). The contribution of thymopoiesis to T cell reconstitution was evaluated based on the frequency of naive CD8⁺CD45RA⁺CCR7⁺ T cells \((45)\) and CD4⁺CD45RA⁺CD31⁺ T cells \((46)\) \((Fig. 1B, 1C)\). Frequencies of naive CD8⁺ T cells remained close to baseline in the first 3 mo posttransplantation, did not increase between 2 and 3 mo \((median, 1.74, 4.22, and 4.61\% at 1, 2, and 3 mo, respectively)\), and then increased progressively between 6 and 18 mo \((median, 23.8, 69.8, 74.3, and 74.8\% at 6, 12, 18, and 24 mo, respectively)\) \((Fig. 1B)\). Naive CD4⁺ T cells were barely detectable at 1 mo postransplantation \((median, 0.40\%)\), but their frequency increased slowly between 2 and 6 mo \((median, 1.30, 3.60, and 10.0\% at 2, 3, and 6 mo, respectively)\). Between 9 and 24 mo posttransplantation, roughly half of the CD4⁺ T cell subset consisted of naive cells \((median, 39.5, 49.0, and 54.0\% at 9, 12, and 24 mo, respectively)\) \((Fig. 1C)\).

**Reconstitution of CMV-specific cell-mediated immune responses**

Five of twenty-eight subjects \((17.9\%)\) were CMV seropositive before transplantation, and four of them \((80\%)\) developed CMV DNAemia. One subject \((P7)\) died of CMV-associated interstitial pneumonia. Another \((P12)\) developed an ophthalmic infection and later died of leukemic relapse. There was a strong association between CMV disease and CMV seropositivity \((p < 0.0001, χ² test)\). IFN-γ ELISPOT using a pp65 peptide mixture as Ag was used to assess reconstitution of anti-CMV cell-mediated immunity in 23 of 28 subjects \((9, 45–47)\). No responses were detected at 1 mo post-UCBT, and the frequency of CMV-specific T cells was significantly lower at 2 mo post-UCBT than that observed in healthy controls \((p < 0.05, Kruskal–Wallis test with Dunn multiple comparison test)\) \((Fig. 2A)\). From 2 to 36 mo, CMV-specific immune reconstitution was steady and progressive, increasing from a mean of 29.2 SFU/10⁶ PBMC at 2 mo postransplant to a mean of 307 SFU/10⁶ PBMC at 36 mo postransplant \((Fig. 2A)\).
IFN-γ responses were heterogeneous, with high SFU values observed at all time points. There was no statistically significant difference between the frequency of CMV-specific T cells measured between 12 and 36 mo posttransplantation and that measured in healthy adult controls ($p > 0.05$, Kruskal–Wallis test with Dunn multiple comparison test). In the Kaplan–Meier analysis, only 3.57% of study subjects had recovered significant anti-CMV immunity at 2 and 3 mo posttransplantation. At 6 and 12 mo, 11.6 and 16.3% of subjects produced detectable IFN-γ in response to CMV. Finally, at 36 mo (end of follow-up), 33.5% of subjects tested had developed anti-CMV cell-mediated immunity (Fig. 2B).

CMV DNAemia, with between 7,000 and 138,107 copies/ml plasma, was detected in subjects P7, P9, P12, and P26, who were then treated with ganciclovir. Subject P7, who developed interstitial pneumonia, was withdrawn from the study at 2 mo post-UCBT, when CMV DNAemia reached 90,000 copies/ml, and his anti-CMV IFN-γ response was not assessed. However, the three other CMV-seropositive subjects (i.e., P9, P12, and P26) exhibited strong IFN-γ responses ($\geq 150$ SFU/10^6 PBMC) at all time points post-DNAemia; they did not develop clinical symptoms or recurrent DNAemia following CMV-specific immune reconstitution (Fig. 3A). The sole CMV-seropositive subject in whom CMV DNAemia was not detected (P11) was 5 mo old at the time of serologic typing, hence, the possible presence of maternal Abs; he did not reconstitute CMV-specific cell-mediated immunity in terms of IFN-γ production (data not shown).

Overall, four cases (21.1%) of spontaneous reconstitution of CMV-specific IFN-γ production were observed in 19 CMV-seronegative recipients with no concurrent or previously documented evidence of CMV DNAemia (P4, P5, P13, and P21) (Fig. 3B). The four patients included subject P4, who underwent UCBT for the treatment of acute myeloid leukemia (AML) at age 10 y, 7 mo and exhibited 140 and 590 CMV-specific SFU/10^6 PBMC at 12 and 18 mo posttransplant, respectively; subject P5, who underwent UCBT for the treatment of myelodysplastic syndrome (MDS) at age 4 y, 11 mo and exhibited 1135 CMV-specific SFU/10^6 PBMC at 18 mo posttransplant; subject P13, who underwent UCBT for the treatment of acute lymphoblastic leukemia (ALL) at age 17 y and exhibited 75 CMV-specific SFU/10^6 PBMC at 36 mo posttransplant; and subject P21, who underwent UCBT to treat MDS at 12 mo of age and exhibited 460, 715, 717, and 2000 CMV-specific SFU/10^6 PBMC at 2, 3, 6, and 12 mo posttransplant, respectively. These responses were considerably higher than the mean response observed at that particular time point (Fig. 3B). Study subjects who did not respond to pp65 peptides in IFN-γ ELISPOT were also unresponsive to peptides derived from the CMV IE-1 protein (data not shown), an Ag against which responses were shown by other investigators to correlate with protective immunity (24). CMV-specific T cell responses were confirmed by staining with HLA-A2–pp65495–503 peptide MHC tetramers in HLA-A2+ subjects (Fig. 4).

**CMV-specific cell-mediated immune responses and thymopoiesis**

The impact of CD8+ and CD4+ T cell reconstitution on the reconstitution of CMV-specific cell-mediated immune responses was examined in UCBT recipients (Table II). During the first 6 mo posttransplantation, the frequency of T cells that produced IFN-γ in response to CMV Ags was associated with absolute CD3+, CD4+, and CD8+ T cell counts ($p = 0.0471, p = 0.0419$, and $p = 0.0364$, respectively, Spearman rank-correlation test). The magnitude of the CMV-specific IFN-γ response was also positively correlated with the frequency of naive CD8+ T cells ($p = 0.0152$, Spearman rank-correlation test) (Table II). These results suggest that both CD4+ and CD8+ T cells participate in the generation of IFN-γ in response to CMV-derived peptides in UCBT recipients and that the emergence of naive CD8+ T cells via thymopoiesis leads to stronger responses.

**Reconstitution of VZV-specific cell-mediated immune responses**

Seventeen subjects (60.7%) exhibited a positive VZV serologic status prior to transplantation, including nine subjects who were treated with famiciclovir prophylaxis for 12 mo. Five of seventeen (29.4%) VZV-seropositive subjects developed zoster-associated clinical symptoms, one of whom (P2) later died as a result of leukemic relapse. There was a higher relative risk for developing VZV disease in VZV-seropositive subjects ($p = 0.0236, \chi^2$ test). However, no differences were observed in the incidence of VZV...
Subjects P2, P5, and P13 did not receive famciclovir prophylaxis or not \( (p = 0.5000, \chi^2 \text{ test}). \) Reconstitution of VZV-specific cell responses was examined in 24 study subjects using IFN-\( \gamma \) ELISPOT and Varivax III as a source of VZV Ag. This reconstitution was progressive, going from a mean of 11 SFU/10^6 PBMC at 3 mo posttransplantation to 243 SFU/10^6 PBMC at 36 mo. The frequency of VZV-specific T cells was significantly lower during the first 3 mo in comparison with at 36 mo and with healthy controls \( (p < 0.05, \text{ Kruskal–Wallis test with the Dunn multiple-comparisons test}). \) There was no statistically significant difference between the frequency of VZV-specific T cells measured between 12 and 36 mo posttransplantation and that measured in healthy adults \( (p > 0.05, \text{ Kruskal–Wallis test with the Dunn multiple-comparisons test}). \) Similar to CMV-specific immune reconstitution, heterogeneous SFU values were observed (Fig. 5A). However, the magnitude of VZV-specific responses was significantly smaller than that of CMV-specific responses \( (p = 0.0273, \text{ Wilcoxon rank-sum test}) \) (Figs. 2A, 2C, 5A, 5C). In a Kaplan–Meier analysis, 0.00 and 3.85% of subjects developed VZV-specific IFN-\( \gamma \) production in four subjects who were CMV seronegative pretransplant. CD3^+ T cell counts (filled bars) were measured by flow cytometry, and frequencies of cells producing IFN-\( \gamma \) in response to CMV Ags (open bars) were measured using IFN-\( \gamma \) ELISPOT and expressed as SFU/10^6 PBMC, as described in Materials and Methods. Horizontal arrows represent the periods of time during which patients experienced episodes of positive CMV DNAemia (tested using real-time PCR) and were treated with ganciclovir. nd, not determined.

In the case of subject P2, zoster was chronic, with recurrences of symptoms at 6 and 18 mo post-UCBT and no significant VZV-specific IFN-\( \gamma \) responses (Fig. 6A). At 20 mo post-UCBT, subject P2 died of leukemic relapse. Subject P5, who received anti-VZV IgG and famciclovir at 18 mo post-UCBT, developed a response of 320 SFU/10^6 PBMC, and no recurrence of clinical symptoms was reported (Fig. 6A). Subject P13 received famciclovir for a year after the first clinical manifestation of zoster 3 mo post-UCBT. The disease reappeared at 18 mo post-transplantation, at which time treatment with famciclovir was reintroduced. However, clinical symptoms reappeared at 28 mo. Subject P13 developed a VZV-specific IFN-\( \gamma \) response of 208 SFU/10^6 PBMC at 36 mo, after which no further recurrences of zoster were reported (follow-up has now reached 60 mo). Subjects P15 and P17 received famciclovir for 1 y post-UCBT and developed zoster after discontinuation of treatment at 21 and 13 mo post-transplantation, respectively; however, they did not develop VZV-specific IFN-\( \gamma \) responses. Subject P15 was vaccinated against VZV at 30 mo post-transplantation, and subject P17 developed a VZV-specific IFN-\( \gamma \) response at 21 mo post-UCBT (150 SFU/10^6 PBMC). Neither developed further symptoms of VZV infection.

A total of 10 VZV-seropositive subjects did not develop any detectable IFN-\( \gamma \) responses against VZV Ag, including 6 subjects who did not receive prophylactic treatment (data not shown). There were no significant differences between subjects who received prophylaxis and those who did not with respect to the proportion of subjects with above-threshold levels of anti-VZV effectors at 12 mo posttransplantation \( (n = 16, p = 0.5000, \chi^2 \text{ test}) \) or with respect to the median frequency of cells that produced IFN-\( \gamma \) \( (p = 0.5313, \text{ Wilcoxon rank-sum test}). \)

Spontaneous reconstitution of VZV-specific cell-mediated immune responses \( \text{(i.e., in the absence of clinical symptoms)} \) occurred...
in 3 of 17 (17.6%) VZV-seropositive subjects (P11, P18, and P20) and in 1 of 7 VZV-seronegative subjects (P21, transplanted at 5 mo of age) (Fig. 6B). Subject P11 underwent UCBT for the treatment of familial hemophagocytic lymphohistiocytosis at age 5 mo and exhibited 178, 358, 630, 435, and 860 VZV-specific SFU/10^6 PBMC at 6, 12, 18, 21, and 36 mo posttransplant, respectively.
subject P18 underwent UCBT to treat ALL at age 7 y and exhibited 150, 243, and 215 VZV-specific SFU/10^6 PBMC at 3, 12, and 18 mo posttransplant, respectively; subject P20 underwent UCBT at age 2 y for the treatment of AML and exhibited 520 VZV-specific SFU/10^6 PBMC at 6 mo posttransplant; and subject P21 (see above) exhibited 168 and 418 VZV-specific SFU/10^6 PBMC at 6 and 12 mo posttransplant, respectively. VZV-seropositive subjects were more likely to reconstitute anti-VZV immunity than were VZV-seronegative patients \( (p = 0.0136, \chi^2 \text{ test}) \) (Fig. 6). VZV-specific IFN-\( \gamma \) responses were associated with VZV seroconversion only in patients who experienced zoster (Table III). Finally, the five subjects who suffered from herpes zoster exhibited VZV-specific IFN-\( \gamma \) responses < 150 SFU/10^6 PBMC at the onset or recurrence of disease, whereas all subjects who reconstituted anti-VZV cell-mediated immunity above the detection threshold in the absence of clinical symptoms (i.e., P11, P18, P20, P21) developed responses \( \geq 150 \) SFU/10^6 PBMC \( (p = 0.0013, \chi^2 \text{ test}) \) (Fig. 6).

**VZV-specific cell-mediated immune responses and thymopoiesis**

The impact of reconstitution of the CD4^+ and CD8^+ T cell subsets on VZV-specific cell-mediated immune responses was examined in UCBT recipients from our study group (Table II). During the first 6 mo, VZV-specific IFN-\( \gamma \) SFU numbers were significantly associated with absolute CD3^+, CD4^+, and CD8^+ T cell counts \( (p = 0.0398, p = 0.0169, \) and \( p = 0.0335, \) respectively, Spearman rank-correlation test). In addition, the magnitude of the VZV-specific response in terms of numbers of IFN-\( \gamma \)-producing cells was positively correlated with the frequency of naive (CD45RA^+ CCR7^+) CD8^+ T cells \( (p = 0.0132, \) Spearman rank-correlation test). These results suggest that both CD4^+ and CD8^+ T cell subsets are implicated in IFN-\( \gamma \) production and that the re-emergence of naive CD8^+ T cells through thymopoiesis leads to more robust VZV-specific cell-mediated immune responses in terms of IFN-\( \gamma \) production.

**Discussion**

To better define the kinetics of reconstitution of CMV- and VZV-specific T cell-mediated immune responses in pediatric HSCT recipients, the progressive recovery of CMV- and VZV-specific T lymphocytes was examined over a posttransplantation period of 3 y in 28 UCBT recipients. In lymphodepleted HSCT recipients, thymus-independent (homeostatic proliferation) and thymus-dependent (thymopoiesis) pathways mediate the reconstitution of the T cell compartment. During the first 100–200 d post-HSCT, graft-derived naive T lymphocytes expand peripherally by undergoing lymphopenia-induced proliferation and acquire both effector and memory markers (reviewed in Ref. 53). These processes are also active following UCBT, where naive T cells differentiate into a population of short-lived effectors enriched in cells that express the programmed death 1 inhibitory receptor (42). De novo T cell production becomes progressively detectable from day 100 onward, and it takes 1–2 y for the level of thymus-derived

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**Table II.** Association between frequencies of CMV- or VZV-specific cells producing IFN-\( \gamma \) and absolute CD3^+, CD4^+, and CD8^+ T cell counts and frequencies of naive CD8^+ and CD4^+ T cells during the first 6 mo post-UCBT

<table>
<thead>
<tr>
<th>Virus</th>
<th>Absolute CD3^+</th>
<th>Absolute CD4^+</th>
<th>Absolute CD8^+</th>
<th>Naive CD4^+</th>
<th>Naive CD8^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV</td>
<td>( p = 0.0471 )</td>
<td>( r = 0.3059 )</td>
<td>( r = 0.3210 )</td>
<td>( r = 0.3381 )</td>
<td>( p = 0.0364 )</td>
</tr>
<tr>
<td>VZV</td>
<td>( p = 0.0398 )</td>
<td>( r = 0.3369 )</td>
<td>( r = 0.4095 )</td>
<td>( r = 0.3576 )</td>
<td>( p = 0.0335 )</td>
</tr>
</tbody>
</table>

Frequencies of cells producing IFN-\( \gamma \) in response to CMV or VZV Ags were determined using IFN-\( \gamma \) ELISPOT, as described in Materials and Methods. Absolute cell counts and frequencies were measured by flow cytometry. Statistical significance was tested using the Spearman correlation test; \( p \) values < 0.05 were considered statistically significant.

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**FIGURE 5.** Reconstitution of VZV-specific T cells over the posttransplant period. (A) Frequencies of cells producing IFN-\( \gamma \) in response to VZV Ags were measured using IFN-\( \gamma \) ELISPOT, as described in Materials and Methods. Data are expressed as SFU/10^6 PBMC. ELISPOT positivity was defined as >50 SFU/10^6 PBMC. Error bars represent median and interquartile range. The dashed line represents the threshold value for ELISPOT positivity. (B) Kaplan–Meier analysis of the frequencies of study subjects exhibiting detectable VZV-specific T cell responses in terms of IFN-\( \gamma \) production. ELISPOT positivity was defined as >50 SFU/10^6 PBMC. (C) Representative ELISPOT wells.
In agreement with other studies (8), the first 3 mo posttransplantation were characterized by persistently low CD4+ and CD8+ T cell counts, as well as by the almost total absence of naive CD4+ and CD8+ T cells. These results confirm that homeostatic expansion of graft-derived T lymphocytes is unable to supply the periphery with significant levels of T cells (52) and that the replenishment of the T cell repertoire is ultimately dependent on thymopoiesis.

The dynamics of reconstitution of CD4+ and CD8+ T cell subsets in the first 3 mo post-UCBT was mirrored by the reconstitution of the CMV- and VZV-specific T cell repertoires. Indeed, at 1 mo posttransplantation, none of the study subjects had reconstituted anti-CMV or anti-VZV responses. At 3 mo, CMV-specific responses were observed in two subjects, whereas only one had regained VZV-specific responses. Such an absence of IFN-γ production in response to these viruses is consistent with the high risk for developing opportunistic viral infections in the first 100 d posttransplantation and with the high frequency of T cells that express programmed death 1 during this period (42). The correlations observed between levels of CMV- and VZV-specific T cells, total CD4+ and CD8+ T cell counts, and naive CD8+ T cell frequencies during the early posttransplantation period indicate that both CD4+ and CD8+ T cells participate in the genesis of IFN-γ responses against these viruses and that thymopoiesis significantly contributes to the regeneration of the antiviral T cell repertoire. Higher levels of IFN-γ production are associated with polyfunctionality of CD8+ T cells and better control of chronic viral infection (55). As a result, the association between the frequency of naive CD8+ T cells and the magnitude of anti-CMV and anti-VZV responses in the first 6 mo post-UCBT implies that de novo production of CD8+ T cells by thymopoiesis (as opposed to UCB-derived T cell expansion) may be required to develop protective responses. These results are in accordance with a recent study showing that thymopoiesis was necessary for the development of strong and protective responses against CMV (29).

Moving away from the 100-d window, the intermediate to late (6–36 mo) posttransplant period was characterized by a gradual increase in levels of T lymphocytes. Reflecting T cell reconstitution, the number of subjects in whom CMV- and VZV-specific responses were detectable increased between 6 and 36 mo posttransplantation, but the respective kinetics of reconstitution of responses against these two pathogens were not significantly different. This is despite the reported differences in timing of CMV-associated (early) versus VZV-associated (late) infectious complications relative to the time of transplant, perhaps reflecting the differential efficacy of cell-mediated immunity in controlling these two particular pathogens (8, 9, 12–14, 31).

Table III. Comparison of VZV-specific IFN-γ and Ab production

<table>
<thead>
<tr>
<th>Subject</th>
<th>Positive IFN-γ</th>
<th>Positive IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13</td>
<td>12–36 mo</td>
<td>12–36 mo</td>
</tr>
<tr>
<td>P15</td>
<td>36 mo</td>
<td>24–36 mo</td>
</tr>
<tr>
<td>P16</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>P17</td>
<td>6–36 mo</td>
<td>12–36 mo</td>
</tr>
<tr>
<td>P18</td>
<td>3–18 mo</td>
<td>Negative</td>
</tr>
<tr>
<td>P26</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>P27</td>
<td>Negative</td>
<td>21–36 mo</td>
</tr>
</tbody>
</table>

Frequencies of cells producing IFN-γ in response to VZV Ags were determined using IFN-γ ELISPOT, and Ab responses were measured by ELISA, as described in Materials and Methods. ELISPOT positivity was defined as >50 SFU/10^6 PBMC.

*Subject developed zoster during the posttransplant period.
protecting graft recipients against these infections. Four UCBT recipients experienced CMV DNAemia, including two cases of symptomatic reactivation. In all instances, DNAemia occurred in the first 6 mo post-UCBT. In three subjects, IFN-γ ELISPOT performed after the DNAemia episode showed the presence of anti-CMV T cell responses in terms of IFN-γ production. Similar results were obtained whether pp65-specific or IE-1–specific responses were examined. No CMV-related events were observed in study subjects as a whole following the development of CMV-specific responses > 150 SFU/10⁶ PBMC. This suggests that CMV DNAemia promotes the development of CMV-specific immunity and that these responses are associated with protection from subsequent CMV-associated complications in UCBT recipients. Frequencies of CMV-specific T cells that produce IFN-γ in response to pp65 peptides range between 0.04 and 4.5% in healthy seropositive individuals, and such frequencies are generally considered to be protective against viral reactivation (56–58). In the current study, the median frequency of CMV-specific T cells measured in healthy adult controls was 453 SFU/10⁶ PBMC (Fig. 2).

Five of twenty-eight subjects developed herpes zoster during the study. The window during which VZV reactivation was observed was wider than that of CMV, extending from 1 mo (subject P13) to 21 mo (subject P15). One subject (P2) never developed IFN-γ responses above the detection threshold and experienced several recurrences of zoster. Another subject (P13) experienced zoster, despite the presence of VZV-specific responses at levels above the detection threshold (≥50 SFU/10⁶ PBMC). However, attainment of IFN-γ responses ≥ 150 SFU/10⁶ PBMC was associated with the absence of any further recurrences of herpes zoster. These results suggest that symptomatic reactivation of VZV does not always lead to the development of VZV-specific responses and that IFN-γ production is protective only beyond a certain threshold (≥150 SFU/10⁶ PBMC). In the case of VZV, frequencies of T cells that produce IFN-γ in response to IE-62 or IE-63 peptide mixes or VZV culture lysate are known to range between 0.03 and 2.75%, and these levels are also thought to be protective (38, 59, 60). In the current study, the median frequency of VZV-specific T cells measured in healthy adult controls was 134 SFU/10⁶ PBMC (Fig. 5).

Importantly, reconstitution of CMV- and VZV-specific T cell responses in the absence of clinical symptoms or episodes of DNAemia was observed in both seropositive and seronegative subjects, although it was less frequent in the latter. This is consistent with previous observations by Cohen et al. (61), although these investigators only relied on proliferative responses that do not necessarily reflect the presence of functional virus-specific T cells. Development of anti-CMV responses in the absence of detectable viral replication was confirmed by staining with MHC–peptide tetramers. Reports showed that CMV infection can occur in CMV-seronegative patients receiving CMV-seronegative grafts, with an incidence ranging from 1.2 to 12% in the first 3 mo posttransplantation (10, 13, 22, 62). However, to our knowledge, kinetics of antiviral immune reconstitution were never assessed in CMV-seronegative patients receiving CMV-seronegative grafts. De novo infection with CMV (i.e., posttransplantation) cannot be excluded as an explanation for the recovery of CMV-specific responses, particularly in the case of subjects P4, P5, and P13, who were older at the time they underwent UCBT. Alternatively, these responses might have arisen as a result of localized intestinal CMV infection, which is sometimes not detected by PCR in peripheral blood (63). Another explanation is that the subjects were falsely seronegative, perhaps as a result of immunosuppression associated with the underlying blood disorders, of borderline Ab titers (64), or of recently acquired or occult CMV infection (65–67). Interestingly, the magnitude of the reconstitution of CMV-specific IFN-γ responses in these four CMV-seronegative subjects was roughly inversely proportional to their age at the time of transplant. Although this number of subjects is too small to draw solid conclusions, it is tempting to speculate that the magnitude of this spontaneous reconstitution of CMV-specific T cell responses was somehow correlated with the extent of residual thymopoiesis in these children, consistent with the existence, generation, and selection of a public CMV-specific T cell repertoire (68, 69).

Following primary infection, VZV is known to remain latent in trigeminal and dorsal root ganglia (70), and its dissemination is thought to be kept in check by host cell-mediated immune responses. In the case of VZV-seropositive subjects P11, P18, and P20, subclinical VZV reactivation might have led to rapid expansion of VZV-specific T cells, thereby preventing the ensuing appearance of herpes zoster. In the case of VZV-seronegative subject P21, it is possible that reconstitution of the VZV-specific T cell repertoire was caused by recently acquired or occult VZV infection or resulted from discordant serologic and cell-mediated immune responses (71–73). Another possibility is that the observed responses are due to antigenic cross-reactivity with another herpes virus (74–77). Alternatively, three of these four subjects were younger than 2 y of age, again suggesting that thymopoiesis might have played an important part in the spontaneous reconstitution of the VZV-specific T cell compartment. Reconstitution of T cell–mediated IFN-γ responses directed against VZV in the absence of clinical symptoms contradicts a recent report on recipients of T cell-depleted BMT (41). However, consistent with our results, Malaviye et al. (78) showed that VZV-specific T cell responses were detectable in patients with malignancies without clinical signs of VZV reactivation, but they appeared weaker or impaired in subjects with detectable VZV viremia. Other investigators showed that VZV-specific T lymphocyte proliferation was detectable in BMT recipients, irrespective of whether they developed symptomatic recurrences of VZV (79), as well as that subclinical VZV viremia can be controlled by T cell-mediated immune responses (80). Taken together, the results reported in this article indicate that clinical or virological manifestations of CMV and/or VZV infection are not a strict requirement for the reconstitution of cell-mediated immune responses directed against these viruses. Spontaneous reconstitution was associated with comparatively strong IFN-γ responses (>150 SFU/10⁶ PBMC), suggesting that UCBT recipients can recover potentially protective CMV- and VZV-specific T cell responses in the absence of symptomatic infection or reactivation. Reports showed that CD8+ T cell reconstitution was more prompt in pediatric BMT recipients compared with UCBT recipients (52). Further studies should assess potential differences between BMT and UCBT with respect to the dynamics of reconstitution of antiviral cell-mediated immune responses.

Finally, we sought to determine whether antitherpetic prophylaxis influenced the development of VZV-specific immunity. In the current study, four of seven VZV-seropositive subjects who exhibited above-threshold frequencies of IFN-γ–producing T cells following stimulation with VZV Ags had been administered famciclovir prophylaxis. In two of them (P15, P17), zoster developed only after interruption of prophylaxis. Thus, famciclovir did not actually prevent the development of herpes zoster but delayed its onset. Levels > 150 SFU/10⁶ PBMC were attained in one nonzoster VZV-seropositive subject (P18; 170 SFU/10⁶ PBMC) during the course of prophylaxis. Therefore, famciclovir prophylaxis did not strictly prevent the reconstitution of the VZV-specific T cell repertoire. Long-term use of famciclovir could have led to the emergence of antiviral drug resistance and ensuing...
VZV, reconstitution

CMV- and VZV-specific T cell responses can also take place in the reconstitution of antiviral immune responses is slow in the majority of UCBT recipients over a period of 3 y. The pattern of antiherpetic immune responses in UCBT recipients could yield key information for the adjustment of immunosuppressive regimens and antiviral prophylaxis.

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Disclosures

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