Patient risk stratification and tailored clinical management of post-transplant CMV-, EBV-, and BKV-infections by monitoring virus-specific T-cell immunity

Anastasia Papadopoulou1 | Kiriakos Koukoulas1,2 | Maria Alvanou1 | Vassilios K. Papadopoulos3 | Zoe Bousiou1 | Vasiliki Kalaitzidou1 | Fotini S. Kika1 | Apostolia Papalexandri1 | Despina Mallouri1 | Ioannis Batsis1 | Ioanna Sakellari1 | Achilles Anagnostopoulos1 | Evangelia Yannaki1,4

1 Hematology Department-Hematopoietic Cell Transplantation Unit, Gene and Cell Therapy Center, “George Papanikolaou” Hospital, Thessaloniki, Greece
2 Department of Genetics, Development and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece
3 Blood Bank Department, General Hospital of Pella-Giannitsa, Giannitsa, Greece
4 Department of Medicine, University of Washington, Seattle, Washington, USA

Abstract

**Background:** Despite routine post-transplant viral monitoring and pre-emptive therapy, viral infections remain a major cause of allogeneic hematopoietic cell transplantation-related morbidity and mortality.

**Objective:** We here aimed to prospectively assess the kinetics and the magnitude of cytomegalovirus-(CMV), Epstein Barr virus-(EBV), and BK virus-(BKV)-specific T cell responses post-transplant and evaluate their role in guiding therapeutic decisions by patient risk-stratification.

**Study design:** The tri-virus-specific immune recovery was assessed by Elispot, in 50 consecutively transplanted patients, on days +20, +30, +60, +100, +150, +200 post-transplant and in case of reactivation, weekly for 1 month.

**Results:** The great majority of the patients experienced at least one reactivation, while over 40% of them developed multiple reactivations from more than one of the tested viruses, especially those transplanted from matched or mismatched unrelated donors. The early reconstitution of virus-specific immunity (day +20), favorably correlated with transplant outcomes. Expanding levels of CMV-, EBV-, and BKV-specific T cells (VSTs) post-reactivation coincided with decreasing viral load and control of infection. Certain cut-offs of absolute VST numbers or net VST cell expansion post-reactivation were determined, above which, patients with CMV or BKV reactivation had >90% probability of complete response (CR).

**Conclusion:** Immune monitoring of virus-specific T-cell reconstitution post-transplant may allow risk-stratification of virus reactivating patients and enable patient-tailored treatment. The identification of individuals with high probability of CR will minimize unnecessary overtreatment and drug-associated toxicity while allowing candidates for pre-emptive intervention with adoptive transfer of VSTs to be appropriately selected.
1 | INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative treatment for hematological disorders. Among its main hurdles however, is the profound T-cell deficiency, resulting in development of viral infections - most commonly by cytomegalovirus (CMV), Epstein Barr virus (EBV) and polyomavirus type I (BKV) - and the substantial transplant-related morbidity and mortality [1,2]. The reconstitution of antiviral immunity post-allo-HCT is often delayed or/and severely impaired by the immunosuppression administered to prevent or treat the immunological complications of allogeneic transplantation, affecting both the quantity and the quality of virus-specific T-cells (VSTs) [3]. The importance of a specific and robust anti-viral immune reconstitution is emphasized by the fact that strategies boosting antiviral immunity, such as immunotherapy with donor- or third-party-derived VSTs, have provided protection with 70–90% response rates [1, 4–7].

Heretofore, clinicians relied exclusively on the viral load monitoring, to guide interventions in treating viral infections post-transplant. Given that immunity is a dynamic process, close monitoring of VST immune reconstitution (VST-IR), may identify patients able to potentially self-control viral reactivations and, eventually, provide the basis for tailored management of antiviral therapy to those in real need avoiding unnecessary overtreatment or/and the outgrowth of drug resistant viral variants.

As opposed to single-virus-specific immune reconstitution, the recovery of viral immunity against multiple viruses has been limitedly investigated in HCT [8,9]. We here aimed, by concomitant monitoring of IR against the most common viruses post-allo-HCT, namely CMV, EBV, and BKV, to prospectively recognize correlations between viral reactivation and the timing and kinetics of VST-IR, compare the recovery of functional VSTs with virological outcomes and provide surrogate markers for identifying patients able to successfully clear the infection and fine-tuning the clinical-decision making.

2 | MATERIALS AND METHODS

2.1 | Subjects

Fifty consecutive allo-HCT patients were included in this prospective study, approved by the Institutional Review Board of the George Papanikolaou Hospital and performed in accordance with the Declaration of Helsinki.

2.2 | Virological monitoring

CMV and EBV viral loads were routinely monitored by quantitative PCR (Qiagen) on blood samples, once a week. BKV load was measured by quantitative PCR (GeneProof) in urine every other week at the presence of clinical signs/symptoms or/and at request of the treating physician.

2.3 | Definitions

CMV/EBV reactivation/infection was defined as increasing viremia with >500 copies/mL in two consecutive measurements or >1000 copies/mL in a single screenshot. BKV reactivation/infection was defined as viruria with >10⁷ copies/mL. Virus detection in tissue fluid or sample accompanied by clinical symptoms was defined as viral disease.

Virus reactivating patients were classified as (i) complete responders (CRs) if there was a return of viral load to normal range and resolution of clinical signs/symptoms, (ii) partial responders (PRs) if there was at least 50% decrease in viral load from baseline or 50% clinical improvement, (iii) non-responders (NRs) when changes were insufficient to qualify as CR or PR.

2.4 | Immunological monitoring by enzyme-linked immunospot

Immunological monitoring was performed in peripheral blood samples collected at days +20, +30, +60, +100, +150, +200 post-allo-HSCT and in case of viral reactivation, weekly for 1 month, as shown in Figure S1. Peripheral blood mononuclear cells (PBMCs) were stimulated with peptides spanning CMV and EBV antigens whereas BKV-STs were firstly expanded in culture (Supplementary Information) [4,5,10], due to their low frequency in blood. PBMCs or expanded BKV-STs were pulsed with viral peptides (CMV: IE1, pp65; EBV: EBNA1, LMP2, BZLF1; BKV: LT, VP1; JPT Peptide Technologies), and IFN-γ secretion was measured by enzyme-linked immunospot (Mabtech). Spot-forming cells (SFCs) were counted on Eli.Scan (A.EL.VIS) using Eli.Analyse software V6.2.SFC. Response, expressed as SFCs per input cells, was considered specific if total SFCs against viral antigens were $\geq 30/5 \times 10^5$ PBMCs or $2 \times 10^5$ BKV-expanded cells.
2.5 | Statistical analysis

Analysis of viral reactivations according to donor type was based on count methods, since each patient could have more than one reactivation event. Matched sibling donors (MSDs) used as the reference category and for each donor sub-group, incidence rates (IR) were calculated as total number of events per 10,000 person-days (equivalent to “per 100 patients per 100 days”) of observation. Byar’s approximation method was used for the IR confidence intervals (CIs). Differences between groups were assessed with IR Ratios (CIs according to Wald method) and chi-square tests, calculated using median-unbiased estimation and exact methods (mid-p). This type of analysis does not take into account the timing of the events, (whether they occur earlier or later during the follow-up) but only the total count of observed events. Analysis was performed in R programming environment with epitools v0.5–10.1 package.

In order to identify factors associated with VSTs, univariate analysis was performed with non-parametric methods (Kruskal-Wallis tests) because their numbers were not normally distributed. Multivariate analysis was performed with multiple linear regression model; VSTs were log-transformed, in order to meet the model’s assumptions.

Non-relapse mortality (NRM) and timing of reactivation events were analyzed with survival methods. Cumulative incidence of reactivation and NRM were calculated with the competing risk model, where death or death from other causes, respectively, was considered a competing event. Comparisons between groups were made with Gray’s test, a modified chi-square test, while survival curves were drawn according to Kaplan-Meier method. Analyses and curves were performed in the R programming environment (v3.6.1) with “survival” and “cmprsk” statistical packages.

To assess whether CR of viral reactivation was correlated with the VST kinetics and could be predicted based on certain cut-offs VST numbers or of the net increase of VSTs from the onset of reactivation up to 2 weeks later (Delta-SFC [$\Delta$SFC:max-VSTs minus onset-VSTs]), a receiver operating characteristic (ROC)-curve analysis was performed. The reactivating patients’ cohort was then split at the optimal VST cut-off, and cumulative incidences of CR between the above or below cut-off sub-cohorts were compared with the competing risk model.

Data are presented as mean ± SEM or median (range) when values follow normal or abnormal distribution, respectively. ANOVA followed by Tukey’s test or a nonparametric Mann-Whitney U test were used for analysis of differences between data sets or multiple comparisons, respectively.

3 | RESULTS

3.1 | Patient characteristics

Fifty adult patients, who consecutively received allo-HCT, from MSD, matched unrelated donor (MUD), mismatched unrelated donor (MMUD), and haploidentical donor (haplo), were enrolled in the study. Haplo-transplant recipients receiving other than unmanipulated bone marrow cells and post-transplant Cy (PTCy) were not included in the study ($n = 2$). Acute graft-versus-host-disease prophylaxis included antithymocyte globulin (ATG), PTCy, Cyclosporin/Methotrexate, Mycophenolate Mofetil; due to ATG’s dominant immunomodulating effect, patients were classified as receiving or not, ATG (Table 1).

Viral prophylaxis in all patients included administration of acyclovir/valacyclovir (lentermovir as primary anti-CMV prophylaxis became available later during the study). Pre-emptive treatment for CMV and EBV antigenemia included ganciclovir or foscarnet and rituximab, respectively. BKV hemorrhagic cystitis was treated with cidofovir. All patients received anti-viral treatment, except otherwise indicated.

3.2 | Viral episodes, IRs, and association with transplant outcomes

From the 50 study subjects, 37 developed at least one viral reactivation from CMV, EBV, and BKV, reaching a total of 78 reactivations (28, 33, 17 CMV, EBV, BKV infections, respectively) through 200 days post-HCT. Fourteen reactivations were diagnosed as or progressed to viral disease (CMV:2 [retinitis, pneumonitis], EBV:1 [encephalitis], BKV:11 [hemorrhagic cystitis]; Table S1), from which one (EBV encephalitis) led to death. The median time to reactivation from any of the three viruses was 35 days (CMV: 37 days [−2 – 164], EBV: 28 days [7–196], BKV: 47 days [21–115]). The highest incidence of viral reactivations (67%) was observed by day 30 post-transplant (22%, 35%, 10% for CMV, EBV, BKV, respectively), while the overall incidence substantially declined by days 100 (20%) and 200 (10%) (Figure S2). The decrease in the incidence of viral reactivations over time, was inversely correlated with VSTs reconstitution (Figure S3).

More than 40% of the patients (21/50) presented multiple reactivations, with a median of two (1–6) reactivations per affected patient (Figure 1A). The MSD-group experienced less infectious episodes over MUD ($p < 0.003$), MMUD ($p = 0.002$), and haplo ($p = 0.13$) presenting the lowest IR ratio (95% CI, 1 vs 2.5 vs 3 vs 2, respectively) (Figure 1B; S4 Table S2). The lower overall infection rate in the MSD group coincided with earlier VST-IR over the other donor groups (Figure 1B; S5).

CMV seropositive recipients demonstrated increased (45.7% vs 23.1%), albeit not statistically significant, incidence of CMV reactivations ($p = 0.15$) over the seronegative hosts, given the constraints of a relatively small number of patients assessed in each group (Table S3). By looking at the kinetics of VSTs, at different time points and in association with the recipient serostatus, we observed significantly earlier and higher CMV-specific immune reconstitution between days +30 and +100 in seropositive over seronegative hosts, probably reflecting boost immune responses to the increased rate of reactivations in seropositive patients (Figure S6). Similar, albeit not significant, kinetics of EBV-ST reconstitution was observed in EBV seropositive recipients (Table S3, Figure S6).

At a median follow-up of 284 days (range 56–592), the NRM was 30% and significantly associated with the occurrence of viral
TABLE 1  Clinical characteristics of the study population

| Patients, n | 50 |
|------------|----|
| Age, median years (range) | 40.5 (17–69) |
| Sex, n (%) |    |
| Male | 30 (60) |
| Female | 20 (40) |
| Diagnosis, n (%) |    |
| Acute myeloblastic leukemia | 21 (42) |
| Acute lymphoblastic leukemia | 13 (26) |
| Myelodysplastic syndrome | 5 (10) |
| Myeloproliferative neoplasms | 4 (8) |
| Hodgkin lymphoma | 3 (6) |
| Chronic lymphocytic leukemia (CLL) | 2 (4) |
| Non-Hodgkin lymphoma | 1 (2) |
| Multiple myeloma | 1 (2) |
| Conditioning regimen, n (%) |    |
| Myeloablative | 38 (76) |
| RIC | 12 (24) |
| Donor type, n (%) |    |
| Matched sibling donor | 17 (34) |
| Matched unrelated donor | 20 (40) |
| Mismatched unrelated donor | 8 (16) |
| Haploidentical | 5 (10) |
| Stem cell source, n (%) |    |
| Bone marrow | 5 (10) |
| Peripheral blood stem cells | 45 (90) |
| Donor/recipient CMV serostatus, n (%) |    |
| Positive/positive (D+/R+) | 28 (56) |
| Negative/positive (D-/R+) | 7 (14) |
| Positive/negative (D+/R-) | 7 (14) |
| Negative/negative (D-/R-) | 6 (12) |
| Unknown | 2 (4) |
| Donor/recipient EBV serostatus, n (%) |    |
| Positive/positive (D+/R+) | 34 (68) |
| Negative/positive (D-/R+) | 5 (10) |
| Positive/negative (D+/R-) | 4 (8) |
| Negative/negative (D-/R-) | 3 (6) |
| Unknown | 4 (8) |
| Acute GvHD, n (%) |    |
| grade 0–I | 38 (76) |
| grade II–IV | 12 (24) |
| ATG-based GvHD prophylaxis or treatment, n (%) |    |
| ATG | 35 (70) |
| No ATG | 15 (30) |

Abbreviations: ATG, anti-thymocyte globulin; CMV, cytomegalovirus; EBV, Epstein-Barr virus; GvHD, graft-versus-host disease GvHD; RIC, reduced intensity conditioning.

reactivations; 15 of 38 patients (40%) who developed at least one reactivation succumbed to transplant complications, whereas none of the 12 (0%) non-reactivating patients died from other than relapse causes ($p = 0.01$; Figure 2A). The higher NRM among virus reactivating over virus non-reactivating patients resulted in lower OS (52.7%, Δm 12 months vs 91.7%, Δm not reached, $p = 0.03$) (Figure 2B). Viral reactivations had a limited impact on relapse rates ($\geq 1$ reactivation vs no reactivation: 10.5% vs 8.3%, $p = ns$; Figure 2C).

3.3 | Viral reactivation and VST-IR

To better understand the temporal relationship between viral reactivations and VST-IR, we compared the CMV- and EBV-ST levels at reactivation onset between recipients who developed infection as per the definition and ”low-viremic” patients who experienced CMV and EBV DNAemia without reaching the threshold values above which viremia was considered infection and treated. The low-viremic patients had significantly higher numbers of cytokine-producing VSTs at reactivation onset than those who ultimately developed viral infection (Figure 3A), suggesting that functional VSTs protected patients against the development of relevant infections. Notably, VSTs markedly expanded from baseline in all viremic patients relative to non-viremic subjects (Figures 3B and 3C), suggesting that antigen stimulation triggers VST proliferation.

3.4 | Risk factors for impaired VST-IR

To identify risk factors for delayed or/and impaired VST-IR, the VST levels at an early time point (day +20), were checked against different variables in univariate and multivariate analysis. ATG administration was strongly associated with significantly lower CMV-, EBV-, and BKV-ST levels both in the univariate and multivariate analysis whereas donor type (mismatched vs matched) did not retain significance in the multivariate analysis (Figure 4). Notably, steroid administration raised as a contributing factor toward delayed reconstitution of EBV-specific T-cell immunity only in the multivariate analysis, probably due to a confounding effect of the other covariates in the univariate analysis.

3.5 | VST-IR and clinical outcome

VST-IR correlated with the clinical outcome; the majority of patients not developing VSTs failed to control infection, while patients with VST rebounds either cleared the infection or developed only low viremia. This positive correlation between VST-IR and successful viral control after allo-HCT and vice versa is shown in three representative cases. The first patient reactivated EBV but did not develop detectable EBV-specific response and succumbed to EBV encephalitis despite receiving rituximab. The second, who rapidly amplified EBV-STs, effectively controlled the viral burden. The third patient who reactivated BKV and did not develop sustained presence of BKV-STs, suffered from prolonged
FIGURE 1  Viral reactivations (A) per donor group. Each bar represents the observation time per patient, grouped by donor type. Occurrence of viral reactivation up to day 200 (squares: CMV, circles: EBV, triangles: BKV) or death (‘x’) is marked on the bar. (B) Per donor group in accordance with early (day 20) virus-specific immune reconstitution. The squares with whiskers represent incidence rate of infections from all viruses per type of donor (95% confidence intervals). Incidence rate ratios and p-values were based on exact mid-p methods. The boxplots depict the distribution of total VSTs on day 20 post-allo-HCT for each type of transplant. Outliers are shown as dots.

Abbreviations: BKV, BK virus; CMV, cytomegalovirus; EBV, Epstein Barr virus; Haplo, haploidentical donor; MMUD, mismatched unrelated donor; MSD, matched sibling donor; MUD, matched unrelated donor; SFC, spot forming cells.

FIGURE 2  Cumulative incidence of non-relapse mortality (A), overall survival (B), and relapse (C) according to reactivation status (Kaplan-Meier curves with 95% confidence intervals [grey zones], p-value derived from Cox proportional hazards’ model (B) or modified Cox subdistribution hazards model (A, C)).

hemorrhagic cystitis despite cidofovir treatment (Figures 5A-5C). To better understand the role of VSTs in controlling viral reactivations, reactivating patients were classified based on their response. As regards CMV, CRs presented a massive CMV-ST expansion, followed by a dramatic decrease in viral load and effective control of the reactivation/disease (Figure 5D). In contrast, at reactivation onset, partial and no responders (PRs/NRs) had significantly lower CMV-STs which either did not expand or profoundly delayed to rebound over CRs (p = 0.039), ultimately being unable to control CMV. Likewise, among patients with BKV reactivations, CRs had higher VST numbers at reactivation onset (p = 0.0031) which coincided with strong T-cell responses leading to resolution of BKV replication and shedding, in contrast to PRs/NRs where the limited or delayed recovery of BKV-STs couldn’t control the increasing viral burden (Figure 5E). A similar, albeit less distinct, correlation was observed in patients with EBV reactivations (Figure 5F). When subgrouping only those patients who developed viral diseases (n = 14 in total, Table S1), again CRs (n = 9) presented higher median VST numbers at reactivation onset and
reached higher VST peaks (Δm 100 SFC /2–5 × 10^5 cells [0–1222] and Δm 1,238 SFC/2–5 × 10^5 cells [323–1700], respectively) over PR/NRs (n = 5) (Δm 0 SFC /2–5 × 10^5 cells [0–22] and Δm 242 SFC/2–5 × 10^5 cells [65–1377]) (p = 0.1 and p = 0.04, respectively) (Figure S7).

3.6 | Predicting CRs and guiding therapeutic decision based on certain levels of virus-specific immunity

To determine a threshold for protective VST immunity, thus avoiding overtreatment or stratifying patients to different therapy options, we performed ROC analysis. In CRs, the absolute VST values at reactivation and the net increase (ΔSFC) of VSTs from the onset of reactivation up to 2 weeks later (ΔSFC:max-VSTs minus onset-VSTs) were compared to their counterparts of PRs/NRs. A cutoff ≥ 63 and ≥ 22 SFC/2–5 × 10^5 cells for CMV and BKV respectively, at time of reactivation, was found optimal, allowing discrimination with high sensitivity and specificity of patients who cleared the infection over PRs/NRs (p = 0.04, p = 0.005, respectively; Table 2, Figures 6A and 6B). Moreover, a ΔSFC ≥ 132 CMV and ≥ 156 BKV/2–5 × 10^5 cells, 1–2 weeks post-reactivation was predictive with high sensitivity and specificity of a CR (p = 0.01, p ≤ 0.05, respectively; Table 2, Figures 6C and 6D). Importantly, CMV reactivating patients in whom either of the CR predictive criteria was fulfilled (SFCs ≥ 63 or ΔSFC ≥ 132) had 94% probability of CR as compared to only 40% probability of CR of those having both values below cut-off (HR:4.96, 95% CI 1.42–17.31, p = 0.004) (Figure 6E). More strikingly, BKV reactivating patients reaching either one of the CR predictive cut-offs, had 100% probability of CR as compared to 0% of patients having both SFCs ≤ 21 and ΔSFC ≤ 155 (p = 0.004; HR not provided due to small sample size, Figure 6F).

Although ROC analysis did not provide predictive values for EBV-specific-IR (Figures S8A and S8B), a rapid and considerable EBV-ST expansion during the initial phase of EBV reactivation was suggestive of subsequent endogenous viral control. Indeed, we used EBV-ST expansion as a tool for clinical decision making and patient-tailored management, in three EBV-reactivating patients in whom the EBV-ST levels and viral load were closely monitored from the onset of viral reactivation onwards. Based on a rapid and significant EBV-ST expansion during the initial phase of reactivation (median ΔSFC 2 weeks post-reactivation: 365 SFC/5 × 10^5 cells, range: 107–855), those patients remained under close monitoring without receiving antiviral pharmacotherapy. Notably, in all patients, EBV reactivation was effectively controlled by the spontaneously expanded endogenous EBV-STs without any treatment (representatively depicted in Figure S8C).

4 | DISCUSSION

Despite improvements in anti-viral pharmacotherapy, CMV, EBV, and BKV reactivations remain a leading cause of morbidity and mortality.
**FIGURE 4** Risk factors for impaired virus-specific immunity. Virus-specific T-cells on day 20–30, according to categorical variables. Distribution of VSTs is shown as boxplot/dot plot combination. p-values for univariate analysis (p) are calculated with Kruskal-Wallis non-parametric statistics. Multivariate p-values (pm) are calculated with multiple linear regression on the log-transformed VST values. Significant differences are indicated in bold. Key: donor type; matched (sibling/unrelated) versus mismatched (unrelated/haplo); *multivariate analysis’ p-values estimate differences from reference category (both seronegative)

Abbreviations: aGvHD, acute GvHD; ATG, anti-thymocyte globulin; BKV-STs, BK virus-specific T cells; CMV-STs, cytomegalovirus-specific T cells; cGvHD, chronic GvHD; D, donor; EBV-STs, Epstein Barr virus-specific T cells; GvHD, graft-versus-host-disease; R, recipient; RIC, reduced intensity conditioning.

**TABLE 2** Sensitivity and specificity of threshold levels of VSTs and their corresponding ΔSFC post reactivation

| Value                        | Threshold | Sensitivity (%) | Specificity (%) | Optimal threshold |
|------------------------------|-----------|-----------------|-----------------|-------------------|
| CMV-STs at reactivation      | >54.5     | 68.42           | 85.71           | >62.5 CMV-STs     |
|                              | >62.5     | 68.42           | 100             |                   |
|                              | >68       | 63.16           | 100             |                   |
| BKV-STs at reactivation      | >3        | 87.5            | 80              | >21 BKV-STs       |
|                              | >21       | 87.5            | 100             |                   |
|                              | >47.5     | 75              | 100             |                   |
| CMV-ST ΔSFC post-reactivation| >104      | 73.33           | 71.43           | >131.5 CMV-ST ΔSFC|
|                              | >131.5    | 73.33           | 85.71           |                   |
|                              | >157.5    | 66.67           | 85.71           |                   |
| BKV-ST ΔSFC post-reactivation| >43.5     | 100             | 60              | >155.5 BKV-ST ΔSFC|
|                              | >155.5    | 100             | 80              |                   |
|                              | >263.5    | 83.33           | 80              |                   |

Abbreviations: BKV-STs, BK virus-specific T cells; CMV-STs, cytomegalovirus-specific T cells.
FIGURE 5  Successful viral control associated with robust VST recovery. (A–C) Individual patient graphs of viral load and VST reconstitution. (A) Example of a patient who reactivated EBV but failed to develop EBV-STs and succumbed to EBV encephalitis. (B) Example of a subject who effectively controlled EBV after reactivation and subsequent expansion of EBV-STs. (C) Example of a subject who reactivated BKV, did not develop sustained numbers of BKV-STs, and suffered from severe, painful, and persistent hemorrhagic cystitis. Dotted lines and solid lines illustrate viral loads and VSTs, respectively. (D–F) Expansion of CMV-STs (D), BKV-STs (E), and EBV-STs (F) in association with viral loads between CRs and PRs/NRs. Dotted lines illustrate viral loads and solid lines illustrate VSTs. Black lines illustrate CRs, and grey lines illustrate PRs/NRs. Differences between data sets were analyzed using Mann-Whitney test (*p < 0.043, **p < 0.006, and ****p < 0.0001).

Abbreviations: BKV-STs, BKV-specific T cells; CMV-STs, CMV-specific T cells; CRs, complete responders; EBV-STs, EBV-specific T cells; PRs/NRs, partial and non-responders; SFC, spot-forming cells; wk, week.

following allo-HCT [1, 2]. Unidimensional guidance of prophylactic, pre-emptive, and therapeutic interventions in transplant recipients by monitoring the viral load without also considering the status of virus-specific immunity, often results in unnecessary treatment, treatment-related toxicities, or/and emergence of drug resistance. On the other hand, a diagnostic tool to safely predict the presence of functional cellular immunity and thus the subsequent successful control of viral infections remains elusive. We here, prospectively focused on the timing, kinetics, and magnitude of VST-IR to common post-transplant viruses (CMV, EBV, and BKV), as a means to risk-stratify patients with viral reactivations and fine-tuning the clinical-decision making.

In total, 74% of patients developed CMV or/and EBV or/and BKV reactivations, with a median of two viral episodes/patient. Apart from the substantial morbidity, and in agreement with other reports [11–17], viral reactivations from these three viruses, correlated also with adverse outcomes as regards OS and NRM, thus underscoring the magnitude of the indirect mortality induced by viral infections and their treatment. The huge human and financial cost associated with the management of viral infections post-transplant provides both a scientific and economic rationale for patient risk-stratification, so as to avoid unnecessary treatment in those patients who have acquired functional virus-specific immunity or to proceed with interventions such as VST immunotherapy for those at high-risk.

Although the correlation between VST presence and viral control has been demonstrated post-solid organ transplantation for all three viruses [18–27], reports in the HCT setting mostly have focused on CMV [16,28–56]. In our study, by simultaneously monitoring functional T-cell immunity against a broad spectrum of clinically problematic viruses after allo-HCT, in conjunction with viral load, we showed...
that VST rebounds, even in patients with viral diseases, were associated with marked reductions of the relevant viral loads and a favorable clinical outcome. Further supporting that even subclinical levels of viral replication boost antigen-experienced T-cell reconstitution [8,28], viral reactivating patients (infected or low viremic) demonstrated considerable VST expansion from baseline relative to non-viremic subjects.

The VST-IR was serially measured by the levels of CD3+ CMV-, EBV-, and BKV-STs in PBMCs. Others have proposed monitoring of CD8+ CMV-STs as a prognostic tool to identify allo-HCT patients at high risk for CMV-infections [52, 57], however, by evaluating only CD8+ cells, and given the importance of CD4+ STs in controlling infections [58], the clinical response may be misinterpreted or underestimated.

Hematopoietic stem cell transplant physicians will more accurately guide their therapeutic decisions if immune competence against viruses could be precisely estimated, enabling the discrimination of patients with solid antiviral immunity and high probability of CR from those having low probability of clearing the infection and being dependent on pre-emptive interventions. Several groups, focusing on CMV, have tried to identify VST thresholds predictive of protection from viral reactivation [59–62]. Instead, we here provide, a prediction model that could identify with at least 94% probability, among CMV and BKV reactivating patients, those who could successfully clear the infection, based on certain VST levels at reactivation or their max ΔSFC 1–2 weeks later. Although ROC analysis couldn’t provide CR predictive cut-offs in EBV reactivating patients, three patients for whom high EBV-VSTs at reactivation were detected, and a considerable expansion was measured in the following 2 weeks, cleared EBV infection without receiving rituximab.

Notwithstanding the need for validation of the predictive cut-offs for viral complete response in a larger, multicenter study, our data stress out the importance of monitoring VST-IR by functional assays that can be used to risk-stratify transplanted patients with viral infections and guide their clinical management. The identification of patients at low risk for morbidity and mortality from viral reactivations will minimize unnecessary overtreatment and drug-associated toxicity. On the other hand, identification of patients at-risk might lead to pre-emptive intervention with intense antiviral pharmacotherapy or virus-specific immunotherapy. Guided pre-emptive therapeutic choices based on the actual individual risk for controlling viral infections will potentially result in lower morbidity and better survival chances in allo-HCT patients as well as substantially decrease the post-transplant care cost.

ACKNOWLEDGMENTS

We would like to thank Nikolaos Savvopoulos, Ioanna Vallianou, and Irene Deligianni (G. Papanikolaou Hospital) for technical assistance.
CONFLICT OF INTEREST
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

FINANCIAL INFORMATION
Funding for this project was provided in part by Research, Technology Development and Innovation (RTDI) State Aid Action “RESEARCH - CREATE - INNOVATE” and in part by a Research Grant award granted by the European Hematology Association.

AUTHOR CONTRIBUTION
Conceptualization: Apostolia Papalexandri and Evangelia Yannaki. Methodology: Apostolia Papalexandri, Kiriakos Koukoulia, Maria Alvanou, Vassilios K. Papadopoulos, Zoe Bousiou, Vasiliki Kalaitzidou, and Anastasia Papadopoulou. Investigation: Apostolia Papalexandri, Kiriakos Koukoulia, Maria Alvanou, Vassilios K. Papadopoulos, Zoe Bousiou, Vasiliki Kalaitzidou, Fotini S. Kika, Anastasia Papadopoulou, Despina Mallouri, Ioannis Batisis, Ioanna Sakellari, and Evangelia Yannaki. Writing – original draft: Apostolia Papalexandri and Evangelia Yannaki. Writing – review and editing: Apostolia Papalexandri, Vassilios K. Papadopoulos, and Evangelia Yannaki. Funding acquisition: Apostolia Papalexandri, Achilles Anagnostopoulos, and Evangelia Yannaki. Resources: Achilles Anagnostopoulos and Evangelia Yannaki. Supervision: Achilles Anagnostopoulos and Evangelia Yannaki.

ORCID
Anastasia Papadopoulou https://orcid.org/0000-0001-5385-8738

REFERENCES
1. Bollard CM, Heslop HE. T cells for viral infections after allogeneic hematopoietic stem cell transplantation. Blood. 2016;127(26):3331–40.
2. Tomblyn M, Chiller T, Einsele H. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. Biol Blood Marrow Transplant. 2009;15(10):1143–238.
3. Ogonek J, Kralj Juric M, Ghimire S. Immune reconstitution after allogeneic hematopoietic stem cell transplantation. Front Immunol. 2016;7:507.
4. Papadopoulou A, Gerdemann U, Katari UL. Activity of broad-spectrum T cells as treatment for AdV, EBV, CMV, BKV, and HHV6 infections after HSCT. Sci Transl Med. 2014;6(242):242ra83.
5. Tzanou I, Papadopoulou A, Naïk S. Off-the-shelf virus-specific T cells to treat BK virus, human herpesvirus 6, cytomegalovirus, Epstein-Barr virus, and adenovirus infections after allogeneic hematopoietic stem-cell transplantation. J Clin Oncol. 2017;35(31):3547-57.
6. Ottaviano G, Chiesa R, Feuchtinger T. Adoptive T cell therapy strategies for viral infections in patients receiving haematopoietic stem cell transplantation. Cells. 2019;8(1):47.
7. Qian C, Wang Y, Reppel L. Virus-specific T-cell transfer from HSCT donor for the treatment of viral infections or diseases after HSCT. Bone Marrow Transplant. 2018;53(2):114–22.
8. Naïk S, Vasileiou S, Aguayo-Hiraldo P. Toward functional immune monitoring in allogeneic stem cell transplant recipients. Biol Blood Marrow Transplant. 2020;26(5):911–9.
9. Saliba RM, Rezvani K, Leen A. General and virus-specific immune cell reconstitution after double cord blood transplantation. Biol Blood Marrow Transplant. 2015;21(7):1284–90.
10. Gerdemann U, Keirnan JM, Katari UL. Rapidly generated multivirus-specific cytotoxic T lymphocytes for the prophylaxis and treatment of viral infections. Mol Ther. 2012;20(8):1622–1632.
11. Rustia E, Violago L, Jin Z. Risk factors and utility of a risk-based algorithm for monitoring cytomegalovirus, Epstein-Barr virus, and adenovirus infections in pediatric recipients after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2016;22(9):1646–53.
12. Admiraal R, de Koning C, Lindemans CA. Viral reactivations and associated outcomes in the context of immune reconstitution after pediatric hematopoietic cell transplantation. J Allergy Clin Immunol. 2017;140(6):1643–50.e9.
13. Teira P, Battiwalla M, Ramanathan M. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: A CIBMTR analysis. Blood. 2016;127(20):2427–38.
14. Hill JA, Mayer BT, Xie H. The cumulative burden of double-stranded DNA virus detection after allogeneic HCT is associated with increased mortality. Blood. 2017;129(16):2316–25.
15. Green ML, Leisenring W, Xie H. Cytomegalovirus viral load and mortality after haemopoietic stem cell transplantation in the era of preemptive therapy: a retrospective cohort study. Lancet Haematol. 2016;3(3):e119–27.
16. Espigado I, De La Cruz-Vicente F, Benmarzouk-Hidalgo OJ. Timing of CMV-specific effector memory T cells predicts viral replication and survival after allogeneic hematopoietic stem cell transplantation. Transpl Int. 2014;27(12):1253–62.
17. Lunde LE, Dasaraju S, Cao Q. Hemorrhagic cystitis after allogeneic hematopoietic cell transplantation: risk factors, graft source and survival. Bone Marrow Transplant. 2015;50(11):1432–37.
18. Yong MK, Lewin SR, Manouel O. Immune monitoring for CMV in transplantation. Curr Infect Dis Rep. 2018;20(4):4.
19. Binggeli S, Egli A, Dickenmann M, Binet I, Steiger J, Hirsch HH. BKV replication and cellular immune responses in renal transplant recipients. Am J Transplant. 2006;6(9):2218–9.
20. Binggeli S, Egli A, Schaub S. Polyomavirus BK-specific cellular immune response to VP1 and large T-antigen in kidney transplant recipients. Am J Transplant. 2007;7(5):1131–9.
21. Schmidt T, Adam C, Hirsch HH. BK polyomavirus-specific cellular immune responses are age-dependent and strongly correlate with phases of virus replication. Am J Transplant. 2014;14(6):1334–45.
22. Schachtner T, Müller K, Stein M. BK virus-specific immunity kinetics: a predictor of recovery from polyomavirus BK-associated nephropathy. Am J Transplant. 2011;11(11):2443–52.
23. Chakera A, Bennett S, Lawrence S. Antigen-specific T cell responses to BK polyomavirus antigens identify functional anti-viral immunity and may help to guide immunosuppression following renal transplantation. Clin Exp Immunol. 2011;165(3):401–9.
24. Ginevri F, Azzi A, Hirsch HH. Prospective monitoring of polyomavirus BK replication and impact of pre-emptive intervention in pediatric kidney recipients. Am J Transplant. 2007;7(12):2727–35.
25. Prosser SE, Orentas RJ, Jurgens L, Cohen EP, Hariharan S. Recovery of BK virus large T-antigen-specific cellular immune response correlates with resolution of bk virus nephritis. Transplantation. 2008;85(2):185–92.
26. Rittà M, Costa C, Sinesi F. Evaluation of epstein-barr virus-specific immunologic response in solid organ transplant recipients with an enzyme-linked immunospot assay. Transplant Proc. 2013;45(7):2754–7.
27. Wilsdorf N, Elz-Vesper B, Henke-Gendo C. EBV-specific T-cell immunity in pediatric solid organ graft recipients with posttransplantation lymphoproliferative disease. Transplantation. 2013;95(1):247–55.
28. Abate D, Cesaro S, Cofano S. Diagnostic utility of human cytomegalovirus-specific T-cell response monitoring in predicting viremia in pediatric allogeneic stem cell transplant patients. Transplantation. 2012;93(5):536–42.

29. Avetisyan G, Larsson K, Aschan J, Nilsson C, Hassan M, Ljungman P. Impact on the cytomegalovirus (CMV) viral load by CMV-specific T-cell immunity in recipients of allogeneic stem cell transplantation. Bone Marrow Transplant. 2006;38(10):687–92.

30. Barron MA, Gao D, Springer KL. Relationship of reconstituted adaptive and innate cytomegalovirus (CMV)-specific immune responses with CMV viremia in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2009;49(12):1777–83.

31. Borchers S, Luther S, Lips U. Tetramer monitoring to assess risk factors for recurrent cytomegalovirus reactivation and reconstitution of antiviral immunity post allogeneic hematopoietic stem cell transplantation. Transpl Infect Dis. 2011;13(3):222–36.

32. Borchers S, Bremm M, Lehrnbecher T. Sequential anticytomegalovirus response monitoring may allow prediction of cytomegalovirus reactivation after allogeneic stem cell transplantation. PLoS One. 2012;7(12):e50248.

33. Clari MA, Muñoz-Cobo B, Solano C. Performance of the QuantIFERON-cytomegalovirus (CMV) assay for detection and estimation of the magnitude and functionality of the CMV-specific gamma interferon-producing CD8(+) T-cell response in allogeneic stem cell transplant recipients. Clin Vaccine Immunol. 2012;19(5):791–6.

34. Giménez E, Muñoz-Cobo B, Solano C. Functional patterns of cytomegalovirus (CMV) pp65 and immediate-early-1-specific CD8(+) T cells that are associated with protection from and control of CMV DNAemia after allogeneic stem cell transplantation. Transpl Infect Dis. 2015;17(3):361–70.

35. Gratawa JW, Corneilissen JJ. Diagnostic potential of tetramer-based monitoring of cytomegalovirus-specific CD8+ T lymphocytes in allogeneic stem cell transplantation. Clin Immunol. 2003;106(1):29–35.

36. Gratawa JW, Boechk M, Nakamura R. Immune monitoring with iTAG MHC Tetramers for prediction of recurrent or persistent cytomegalovirus infection or disease in allogeneic hematopoietic stem cell transplant recipients: a prospective multicenter study. Blood. 2010;116(10):1655–62.

37. Guerrero A, Riddell SR, Storek J. Cytomegalovirus viral load and virus-specific immune reconstitution after peripheral blood stem cell versus bone marrow transplantation. Biol Blood Marrow Transplant. 2012;18(1):66–75.

38. Hakki M, Riddell SR, Storek J. Immune reconstitution to cytomegalovirus after allogeneic hematopoietic stem cell transplantation: impact of host factors, drug therapy, and subclinical reactivation. Blood. 2003;102(8):3060–7.

39. Lee SM, Kim YJ, Yoo KH, Sung KW, Koo HH, Kang ES. Clinical usefulness of monitoring cytomegalovirus-specific immunity by quantiferon-CMV in pediatric allogeneic hematopoietic stem cell transplantation recipients. Ann Lab Med. 2017;37(3):277–81.

40. Lilleri D, Fornara C, Chiesa A, Caldera D, Alessandrino EP, Gerna G. Human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in adult allogeneic hematopoietic stem cell transplant recipients and immune control of viral infection. Haematologica. 2008;93(2):248–56.

41. Lilleri D, Gerna G, Zelini P. Monitoring of human cytomegalovirus and virus-specific T-cell response in young patients receiving allogeneic hematopoietic stem cell transplantation. PLoS One. 2012;7(7):e41648.

42. Mendes AVA, Benard G, Pereira CB. Different kinetics in anti-cytomegalovirus immunity reconstitution evaluated by lymphocyte proliferation and IFN-gamma production in allogeneic and autologous bone marrow transplantation. Acta Haematol. 2002;107(4):187–94.

43. Merindol N, Salem Fourati I, Brito R-M. Reconstitution of protective immune responses against cytomegalovirus and varicella zoster virus does not require disease development in pediatric recipients of umbilical cord blood transplantation. J Immunol. 2012;189(10):5016–28.

44. Moins-Teisserenc H, Busson M, Scieux C. Patterns of cytomegalovirus reactivation are associated with distinct evolution profiles of immune reconstitution after allogeneic hematopoietic stem cell transplantation. J Infect Dis. 2008;198(6):818–26.

45. Nemeckova S, Krystofova J, Babiarova K. Reconstitution of cytomegalovirus-specific T-cell response in allogeneic hematopoietic stem cell recipients: the contribution of six frequently recognized, virus-encoded ORFs. Transpl Infect Dis. 2016;18(3):381–9.

46. Nesher L, Shah DP, Ariza-Heredia EJ. Utility of the enzyme-linked immunospot interferon-γ-release assay to predict the risk of cytomegalovirus infection in hematopoietic cell transplant recipients. J Infect Dis. 2016;213(11):1701–7.

47. Noviello M, Forcina A, Veronica V. Early recovery of CMV immunity after HLA-haploidentical hematopoietic stem cell transplantation as a surrogate biomarker for a reduced risk of severe infections overall. Bone Marrow Transplant. 2015;50(9):1262–4.

48. Ozdemir E, St John LS, Gillespie G. Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. Blood. 2002;100(10):3690–7.

49. Ohnishi M, Sakurai T, Heike Y. Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic hematopoietic stem cell transplantation using interferon-gamma-enzyme-linked immunospot and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope. Br J Haematol. 2005;131(4): 472–9.

50. Pelák O, Stuchlík J, Král L. Appearance of CMV specific T-cells predicts fast resolution of viremia post hematopoietic Stem cell transplantation. Cytometry B Clin Cytom. 2017;92:380–8.

51. Pourghesari B, Piper KP, McLarnon A. Early reconstitution of effector memory CD4+ CMV-specific T cells protects against CMV reactivation following allogeneic SCT. Bone Marrow Transplant. 2009;43(11):853–61.

52. Tey S-K, Kennedy GA, Cromer D. Clinical assessment of anti-viral effectors memory CD4+ T cells in recipients of allogeneic stem cell transplantation using interferon-gamma-ELISPOT assay to identify high risk allogeneic hematopoietic stem cell transplant patients with CMV infection complications. Boussiotsis VA, ed. PLoS One. 2013;8(10):e74744.

53. Torno N, Solano C, Benet I. Lack of prompt expansion of cytomegalovirus pp65 and IE-1-specific IFNγamma CD8+ and CD4+ T cells is associated with rising levels of pp65 antigenemia and DNAemia during pre-emptive therapy in allogeneic hematopoietic stem cell transplant recipients. Bone Marrow Transplant. 2010;45(3):543–9.

54. Torno N, Solano C, Benet I. Reconstitution of CMV pp65 and IE-1-specific IFN-γ CD8(+) and CD4(+) T-cell responses affording protection from CMV DNAemia following allogeneic hematopoietic SCT. Bone Marrow Transplant. 2011;46(11):1437–43.

55. Torno N, Solano C, Benet I. Kinetics of cytomegalovirus (CMV) pp65 and IE-1-specific IFNγamma CD8+ and CD4+ T cells during episodes of viral DNAemia in allogeneic stem cell transplant recipients: potential implications for the management of active CMV infection. J Med Virol. 2010;82(7):1208–15.

56. Zhou W, Longmate J, Lacey SF. Impact of donor CMV status on viral infection and reconstitution of multifunction CMV-specific T cells in CMV-positive transplant recipients. Blood. 2009;113(25):4675–76.

57. Krawczyk A, Ackermann J, Gołtowski B. Assessing the risk of CMV reactivation and reconstitution of antiviral immune response post bone marrow transplantation by the QuantiFERON-CMV-assay and real time PCR. J Clin Virol. 2018;100:61–6.
58. Feuchtinger T, Opherk K, Bethge WA. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood. 2010;116(20):4360–7.

59. Tobin LM, Healy ME, English K, Mahon BP. Human mesenchymal stem cells suppress donor CD4(+) T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease. Clin Exp Immunol. 2013;172(2):333–48.

60. Raeiszadeh M, Pachnio A, Begum J, Craddock C, Moss P, Chen FE. Characterization of CMV-specific CD4+ T-cell reconstitution following stem cell transplantation through the use of HLA class II-peptide tetramers identifies patients at high risk of recurrent CMV reactivation. Haematologica. 2015;100(8):319–22.

61. Rogers R, Saharia K, Chandrokar A. Clinical experience with a novel assay measuring cytomegalovirus (CMV)-specific CD4+ and CD8+ T-cell immunity by flow cytometry and intracellular cytokine staining to predict clinically significant CMV events. BMC Infect Dis. 2020;20(1):58.

62. Bunde T, Kirchner A, Hoffmeister B. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. J Exp Med. 2005;201(7):1031–6.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Papadopoulou A, Koukoulias K, Alvanou M, et al. Patient risk stratification and tailored clinical management of post-transplant CMV-, EBV-, and BKV-infections by monitoring virus-specific T-cell immunity. eJHaem. 2021;2:428–439. https://doi.org/10.1002/jha2.175