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Increased proportion of perforin-expressing CD8+ T-cells indicates control of herpesvirus reactivation in children after stem cell transplantation

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Abstract Hematopoietic stem cell transplantation (HSCT) is frequently complicated by viral reactivations. Early diagnosis of viral reactivations and preemptive therapy relies on frequent viralload monitoring. An easy marker of effective cytotoxicity in lymphopenia is lacking and therefore we studied perforin-expression in CD8+ T-cells in children following HSCT. Prospectively, we weekly monitored viral loads and perforin-expression of CD8+ T-cells in whole blood by FACS, until 4 months after HSCT in children. 27 patients were included (median age 4,3, range 0.3–20,1 years) of whom 19 developed viral reactivations. These patients showed higher percentages of perforin-expressing CD8+ T-cells (17,2%, range 0–63%) than those without (6,8%; range 0–16%) (p = 0.001). The increased percentage of perforin-expressing CD8+ T-cells coincided with a decrease in viral load with a median interval between maximum viral load and maximum level of perforin-expression of 0,4 weeks (range 0.1–7.1). We conclude that perforin-expression in CD8 + T-cells may be a marker for effective antiviral T-cell reconstitution early after HSCT in children.

1. Introduction

Herpesvirus reactivations, like human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) have been associated with acute Graft-versus-Host Disease (acute-GvHD), allograft rejections and increased non-relapse mortality (NRM) after hematopoietic

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stem cell transplantation (HSCT) [1–3]. Recently, also human herpesvirus 6 (HHV6), which reactivates early after HSCT, was found to be associated with acute-GvHD and increased NRM in children and adults [4,15]. Delayed or inadequate immune responses early after HSCT are associated with prolonged viral reactivation and severe complications.

Although the number of lymphocytes increases rapidly during the first months after HSCT, the frequency of viral reactivations and associated disease remains high, indicating poor reconstitution of specific immunological responses [5].

At present it is clear that T-lymphocytes play an important role in the clearance of viral reactivations after HSCT, however an easy marker of effective T-cell responses for predicting outcome of viral reactivations is lacking. Perforin is one of the effector molecules used by CD8+ T-cells and NK-cells to kill virally infected cells. It is present in cytoplasmic granules, which, after activation of effector T cells, are transported to the plasma membrane and secreted in the immunological synapse to form pores in cell membranes of virally infected cells. Others clearly demonstrated that cytotoxicity mediated by CD8+ T-cells in vivo is nearly completely dependent on the perforin/granzyme system [6,7]. Therefore, we studied perforin-expression in CD8+ T-cells and NK-cells in relation to viral reactivations early after HSCT to assess the potential role of perforin as a marker for viral load control.

2. Methods

2.1.1. Study population

In this prospective cohort study all children (0–18 years) who received a first allogeneic HSCT, irrespective of the indication, between January 2007 and April 2008 in the University Medical Center of Utrecht (UMCU) were included. Transplantation characteristics and all transplantation associated morbidities (e.g. Graft-versus-host-disease: GvHD, viral complications) were registered. Patients were enrolled after the patient or parents had provided written informed consent. This study was approved by the Medical Ethics Committee of the UMCU. Additionally, we analyzed whole blood samples from 12 healthy controls (1–28 years).

2.1.2. Transplantation characteristics

All patients received either a chemotherapy (busulfan) based or total body irradiation (TBI) based myeloablative conditioning regimen. Recipients of an unrelated donor received serotherapy with anti-thymocyte globulin (ATG)-rabbit (Genzyme, Cambridge, USA).

Acute-GvHD was diagnosed and graded according to the Glucksberg criteria [8]. Cyclosporine-A (dose targeted on plasma levels of 100–200 μg/L) was given to all patients as GvHD prophylaxis. Recipients of matched sibling donor cells received cyclosporine-A as single agent, in recipients of unrelated marrow donor cells methotrexate (short course: 10 mg/m^2 on day +1, +3 and +6 after HSCT) was added while in cord blood recipients prednisolon (1 mg/kg until day +28 after HSCT, with a taper in 14 days) was added. Immunosuppressive therapy was tapered after 3 months if no acute-GvHD was present.

2.1.3. Viral monitoring

All HSCT patients were weekly monitored by quantitative real-time PCR for plasma DNA-load of EBV, CMV and HHV6 during the first 4 months after HSCT, as previously described [4]. Based on previous studies, a viral reactivation was defined as viral DNA load >1000 cp/mL in 2 or more consecutive samples after HSCT [4,9].

2.1.4. Immunophenotyping

Weekly immunophenotyping was performed until 4 months after HSCT. Total numbers of T-lymphocytes and NK-cells were determined by using TruCount analysis [10]. After erythrocyte lysis, cells were stained with monoclonal antibodies (Mab): CD3 PerCp, CD8 APC-Cy7, CD62L PE, CD56 APC (BD, Biosciences, Franklin Lakes, USA) and CD16 Pacific Blue (eBioscience Inc, San Diego, USA). After permeabilization, cells were stained with Perforin-FITC labeled Mab (BD, Biosciences, Franklin Lakes, USA). At least 100,000 events where acquired on the LSR II flow cytometer and data were analyzed using BD FACSDiva-software (BD, Biosciences, Franklin Lakes, USA).

2.1.5. (Pre-emptive) antiviral therapy

Due to the very high incidence of primary HHV6 infection early in life, we considered all of our patients and donors (except for cord blood grafts) seropositive for HHV6 [11].

Pre-emptive treatment for CMV- and EBV-reactivations was initiated, according to institutional guidelines, when the viral load exceeded 1000 cp/mL. Patients with HHV6-reactivation were treated with foscarnet-sodium in case of high clinical suspicion of HHV6-associated disease (e.g. encephalitis, colitis) [4].

2.2. Statistical analysis

Patients were divided into 2 groups, based on the occurrence of herpesvirus reactivation (HHV6, EBV and/or CMV reactivation) during follow-up. Differences in patient and transplantation characteristics between patients with and without herpesvirus reactivation were analyzed using Chi-square analyses. Differences in proportion of perforin-expressing cells between patients with and without herpesvirus reactivation in the first 4 months after HSCT were analyzed using Mann–Whitney tests with significance defined by $p < 0.05$ using a 2-tailed test. In all analyses, median values were analyzed. All analyses were performed using software program SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Viral reactivations after HSCT

Twenty-seven patients were included with a median follow-up of 15 months (range 1–26). All patients achieved full donor chimerism, except one patient who developed acute graft rejection 6 weeks after HSCT. During the first 4 months after HSCT, 19 patients developed a viral reactivation. 16/27 (59%) patients developed HHV6 reactivation, one patient CMV reactivation, one patient EBV reactivation and one patient suffered from HHV6, CMV and EBV reactivations. No differences in gender, indication (malignant or non-malignant), donor source (cord blood or bone marrow derived stem cells),

donor type (related or unrelated), HLA-matching and conditioning (chemotherapy-based or TBI-based) were observed between the patients with or without herpesvirus reactivation (Table 1). Age at HSCT was higher in patients with herpesvirus reactivations compared to those without (4.7 years, range 0.8–20.1, compared to 1.5 years, range 0.3–9.6, p = 0.029), but no difference in recipient and donor serostatus for EBV and CMV was noted among the groups. Acute-GvHD (≥ grade 2) was observed in 3 of 8 patients without herpesvirus reactivation compared to 5 of 19 patients with herpesvirus reactivation (p = 0.658).

Pre-emptive antiviral treatment was effective in the patients with CMV- or EBV-reactivation, since viral loads decreased <1000 cp/mL. Two of 19 patients with herpesvirus reactivation deceased after HSCT versus 1 of 8 patients without herpesvirus reactivation. Two of 17 (12%) patients with clinical suspicion of HHV6 associated disease (encephalitis, bone marrow suppression) were treated with foscarnet-sodium and recovered from suspected HHV6 disease. One patient with HHV6, CMV and EBV reactivation at the same time did not respond to antiviral treatment and deceased due to disseminated viral disease.

Table 1 Patient characteristics.

Variable	No herpesvirus (n = 8)	Herpesvirus (n = 19)	p-Value [a]
Median age (range)	1.5 (0.3–9.6)	4.7 (0.8–20.1)	0.029
Gender			
Male	3	14	0.102
Female	5	5	
Donor source [b]			
BM	5	10	0.696
CB	3	9	
Mismatch			
No	6	15	0.999
Yes	2	4	
Indication [c]			
Non-malign.	4	5	0.750
Malignant	4	14	
Donor type			
Unrelated	5	15	0.633
Family	3	4	
Conditioning [d]			
Chemo	5	11	0.999
TBI	3	8	
EBV serology (R/D) [e]			
+/+	1	5	0.251
+/−	2	8	
−/+	2	1	
−/−	3	5	
CMV serology (R/D)			
+/+	0	3	0.404
+/−	3	6	
−/+	0	1	
−/−	5	9	

1 patient developed acute graft rejection at week 6 after HSCT.

[a] p-Values calculated with Chi-square analysis or Mann-Whitney U *t*-test.

[b] BM = bone marrow derived stem cells, CB = cord blood derived stem cells.

[c] Non-malign. = non-malignant.

[d] Chemotherapy based conditioning, TBI = total body irradiation.

[e] R = recipient, D = donor.

3.2. Viral reactivation and Perforin-expressing CD8 + T-cells after HSCT

To analyze differences in perforin-expression, patients were divided into two groups according to the presence of herpesvirus reactivation after HSCT. No differences between the 2 groups in the maximum CD3+ CD8+ T-cell and CD3-CD16+ CD56+ NK-cell numbers were noted (Fig. 1).

To analyze the presence of potential cytotoxic T-cells, perforin expression in CD3+ CD8+ T-cells was prospectively measured weekly after HSCT in parallel to the viral load assessments (Fig. 2). In 12 healthy controls (median age 11 years, range 1–28) the proportion of perforin-expressing CD8+ T-cells was <7% of total CD8+ T-cells in all controls (data not shown, previously described by Pietersma et al.) [13]. Six of the 27 HSCT patients (22%) also remained below 7%; five of them did not develop a herpesvirus reactivation.

The median peak in perforin-expressing CD8+ T-cells was 18% (range 0–45, Fig. 3). Patients with herpesvirus reactivation showed a significant higher percentage of perforin-expressing CD8+ T-cells during the decline of viral load (18%, range 8–45%) than those without reactivation (7%, range 0–16%), (Fig. 3). No difference in perforin-expressing CD3-CD16+ NK-cells or CD3-CD56+ NK-cells was found between patients with or without herpesvirus reactivation (Fig. 3).

In the overall reactivating group, the median time to the peak in perforin-expressing CD8+ T-cells was 4.9 weeks (range 1.4–15.9 weeks) after HSCT. In 18/19 (95%) patients with a herpesvirus reactivation, the proportion of perforin-expressing CD8+ T-cells increased and peaked at week 5 (range 2–13) after HSCT (Table 2). This coincided with the decrease in viral load since the time to herpesvirus reactivation peak was 2 weeks (range 1–16) after HSCT (Table 2) and the duration of viral reactivation was 3 (range 0–16) weeks, with a maximum viral load of 8259 (range 1000–16,1000) cp/mL (Table 2). This is illustrated for a representative patient in (Fig. 4A).

In 14 of 18 (78%) patients, the interval between maximum viral load peak and peak in perforin-expressing CD8+ T-cells was 3 days (range 1–11). Four of 18 (22%) patients showed a delayed peak in perforin-expressing CD8+ T-cells (range 20–29 days) after maximum viral load peak, but eventually cleared the viral load after the peak in perforin-expressing CD8+ T-cells (Table 2). Interestingly, one patient suffered from multiple prolonged viral reactivations. This patient showed no peak in perforin-expressing CD8+ T-cells during follow up and the maximum percentage of perforin-expressing CD8+ T-cells was <7% (Fig. 4B).

Notably, the peak in perforin-expressing CD8+ T-cells corresponded with opposite kinetics of CD62L-expression, a marker of lymph-node homing which is down regulated upon antigen-triggering in the lymph nodes. CD62L-expression on CD8+ T-cells decreased from 80% (range 52–100%) to a

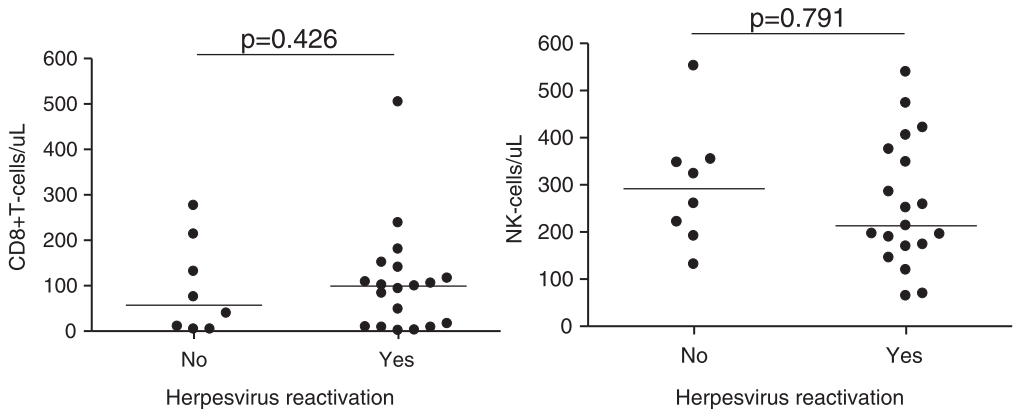

Figure 1 Absolute number of CD8+ T-cells and NK-cells after HSCT. Median number of maximum absolute CD8+ T-cells and NK-cells are plotted for patients with or without herpesvirus reactivation during the first 4 months after HSCT.

minimum of 34% (range 0–71%, Table 2) simultaneously with the peak in perforin-expression after HSCT (5 weeks, range 2–13, Fig. 4), while the high percentage of CD62L-expression on CD8+ T-cells is maintained in the patient with prolonged multiple viral reactivation (Fig. 4). To analyze the association between the peak in perforin-expressing CD8+ T-cells and the decrease in CD62L-expression on CD8+ T-cells, the index was calculated. Interestingly, during clearance of viral reactivation patients showed a higher CD62L/maximal perforin-expression index on CD8+ T-cells (index 0.8, range 0.3–4.4) compared to those without cleared viral reactivation (index 0.2, range 0–0.6, p = 0.006, Fig. 3).

4. Discussion

In this first pediatric study analyzing the reconstitution of perforin-expressing CD8+ T-cells after HSCT in relation to plasma viral reactivation, we found perforin to be a potential marker for predicting effective antiviral activity.

All patients showed a significant peak in perforin expressing CD8+ T-cells during viral load clearance, indicative of an important role of these effector T-cells in control of herpesvirus reactivations. Interestingly, this coincided with a drop in CD62L-expression on CD8+ T-cells, confirming the effector function of these T-cells in control of herpesvirus reactivation after HSCT. Although the majority of patients in our study developed HHV6 reactivation, instead of CMV and EBV reactivations early after HSCT, we and others have previously shown that T-cells are most important in EBV and CMV viral load clearance [9–13]. Although others found that total CD3+ T-cell number after HSCT is a marker for sufficient anti-EBV immune reconstitution, we do not observe differences in absolute T-cell numbers. Instead, we show that perforin-expressing CD8+ T-cells are an easy marker for identifying sufficient anti herpesvirus immune reconstitution. Although not confirmed by other homing markers, the coincidence with a drop in CD62L-expression is suggestive for true effector function of these perforin-expressing CD8+ T-cells. Lack of CD62L identifies effector T-cells which migrate from the

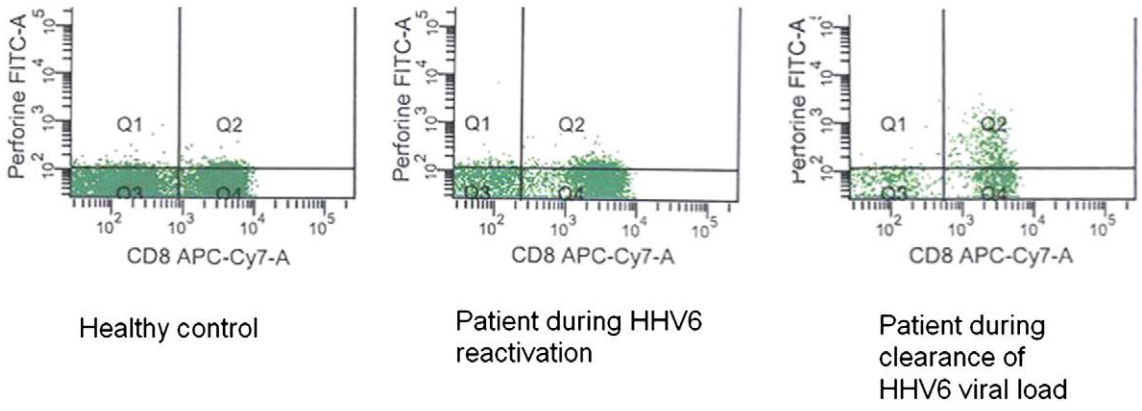

Figure 2 Representative FACS plot of perforin expression in CD8+ T cells. Flow-cytometry dotplot of Perforin-expression (Y-axis) in the CD8+ T cells (x axis) is shown for a healthy control (left panel), a SCT recipient during viral reactivation (middle panel) and a SCT recipient during clearance of viral reactivation (right panel).

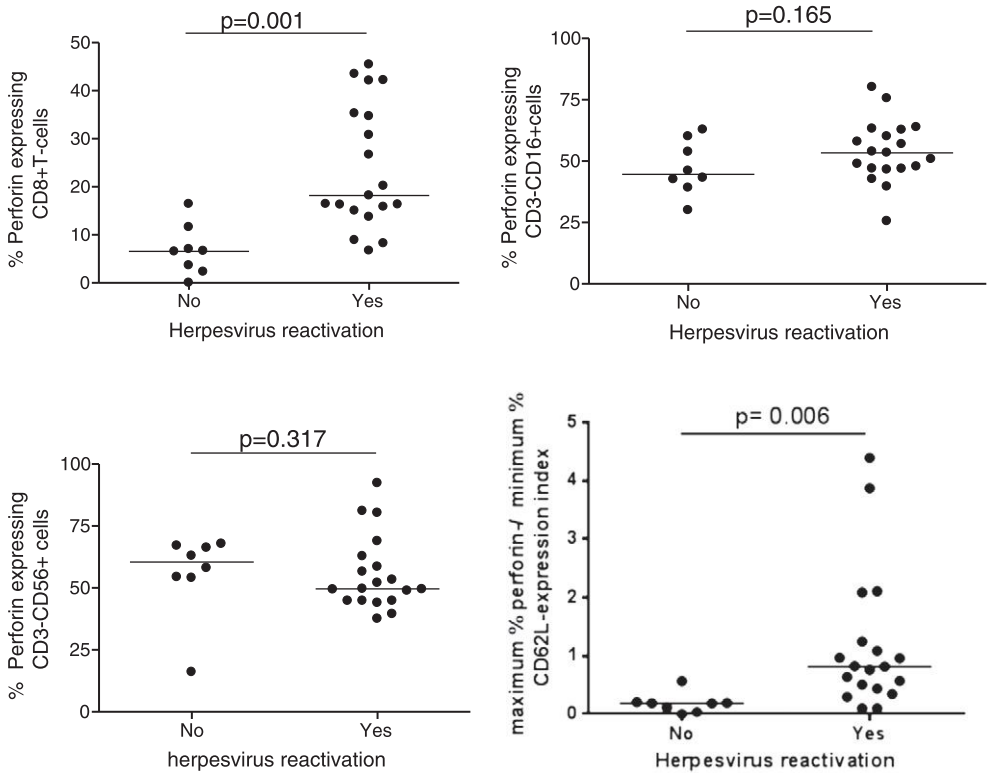

Figure 3 Proportion perforin-expressing cells in children in CD8+, CD16+ and CD56+ cells after HSCT. Median peak of perforin-expressing CD8+ T-cells (upper left panel), CD3- CD16+-NK-cells (upper right panel) and CD3- CD56+ T-cells (lower left panel) is plotted for patients with or without herpesvirus reactivation (in percentages). Perforin-expression was determined by intracellular FACS staining of perforin combined with CD3, CD8, CD16 or CD56 surface staining. The peak in perforin-expressing cells was included for each patient during the first 4 months after HSCT. CD62L expression was determined by FACS staining of CD62L combined with CD3, CD8 surface staining. The index of the peak in perforin-expressing CD8+ T-cells and the decrease in CD62L-expression on CD8+ T-cells during the first 4 months after HSCT were calculated per patient (lower right panel). This was illustrated for patients with cleared viral reactivation and patients without cleared viral reactivation.

lymph nodes to the affected organ(s). In the future, combined analyses with an extended panel of markers may strengthen this evidence. Previously, Annels et al. have shown that an increase in total T-cell numbers was a marker for sufficient anti-EBV activity, but only EBV-specific T-cells were analyzed [12]. These T-cells were measured at a later time point compared to our study and since absolute T-cells numbers are low early after HSCT, the efficacy of these T-cells might be more important than the absolute number.

Both absolute NK-cell numbers, but specifically perforin-expressing NK-cells did not differ among patients with or without herpesvirus reactivation. This may implicate that NK-cells do not play a major role in viral load clearance after HSCT.

We recently performed similar analyses in the adult HSCT setting and surprisingly a high level of perforin-expressing CD8T-cells preceded viral reactivation early after HSCT in adults [13]. There are several important differences in observations between the adult and pediatric HSCT cohort.

First, the adult patients mainly received non-myeloablative conditioning regimen, instead of the total ablative regimen in the pediatric cohort. Since we and others already observed that HHV6-reactivation hardly occurs after nonmyeloablative regimen in adults, this might have strongly influenced the occurrence of early viral reactivation in adults [15]. The viral reactivations of EBV and CMV in adults after HSCT occurred significantly at a later time point after HSCT [13,14]. Secondly, due to the non-myeloablative conditioning regimen, there is a strong graft-versus-host effect shortly after HSCT. This might influence the perforin-peak early after HSCT due to alloreactive phenomenons and may trigger reactivations of latent viruses from immune cells (e.g. EBV from B-cells) at a later time point.

In conclusion, we observed that control of herpesvirus reactivation was associated with a peak in the proportion of perforin-expressing CD8+ T-cells rather than an increase in absolute CD3+ T-cells or (perforin-expressing) NK-cells. This suggests that this lymphocyte-subset marker might indicate specific antiviral immunity in children after HSCT. Although the majority of patients in our study developed HHV6 rather than CMV and EBV reactivation, it is known that T-cells are important in the control of all herpesviruses

Table 2 Characteristics of herpesvirus reactivations.

	Patients with herpesvirus reactivation (n = 19)
Median time to herpesvirus reactivation peak (weeks)	2 (1–16)
Duration of viral reactivation (weeks)	3 (0–16)
Time of maximum proportion of perforin-expressing CD8+ T-cells (weeks)	5 (2–13)
Interval maximum viral load and peak in perforin-expressing CD8+ T-cells (days) [a]	4 (1–29)
Height perforin-expressing CD8+ T-cells at peak (%)	18 (8–45)
CD62L at time of peak perforin-expressing CD8+ T-cells (%)	34 (0–71)

p-Value was calculated using Mann–Whitney tests with significance defined by $p < 0.05$ using a 2-tailed test.

[a] n = 18. 1 patient did not develop a peak in perforin expressing CD8 + T-cells.

after HSCT [12–14]. Thus, the proportion of perforin-expressing CD8+ T-cells is a suitable marker for herpesvirus immunity in pediatric HSCT patients and when confirmed in larger cohort studies, may support the decision-making process of starting antiviral therapy during herpesvirus reactivation after HSCT.

Conflict of interest statement

All authors declare no conflict of financial interest.

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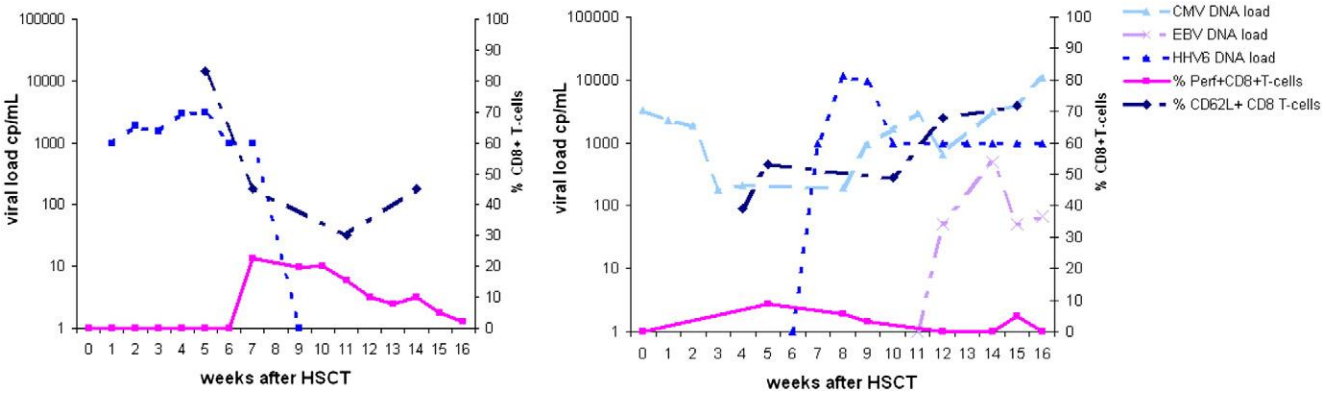

Figure 4 Kinetics of viral reactivation in relation to perforin-expressing CD8 + T-cells. Herpes viral load is plotted in copies/mL (left y-axis) and the percentage of perforin-expressing CD8 + T-cells (right y-axis) is plotted for a representative patient with a peak in perforin-expressing CD8 + T-cells during viral load control (left panel) and a patient with multiple viral reactivations and no peak in perforin-expressing CD8 + T-cells (right panel). Additionally, the percentage of CD62L + CD8 + T-cells is depicted in this figure (right y-axis).

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