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ABSTRACT

Background: CMV, EBV, and ADV infections represent major complications following hematopoietic stem cell transplantation (HSCT). Adoptive immunotherapy using virus-specific T-lymphocytes (VSTs), which are present in the CD45RA-depleted fraction, is a promising alternative, yet accurate quantification of functional VSTs in donor products remains technically challenging. The aim of this study is to assess the detectability and functional activity of VSTs in CD45RA-depleted donor lymphocyte infusion (DLI) products before and after cryopreservation, with particular attention to the effect of cell resting.

Methodology: CD45RA-depleted fractions were obtained from 199 healthy donors. Three sample groups were analyzed: fresh products (n=122), cryopreserved for 2-4 weeks (n=30), and cryopreserved for 5 years (n=15). Cells were rested in CTL Test Medium for 18–24 hours at 37°C

before ELISpot analysis. VSTs specific to CMV, EBV, and ADV were quantified using ELISpot assay. The Mon/CD3 ratio was determined by flow cytometry.

Results: Poor detection of VSTs was observed in CD45RA-depleted samples with a Mon/CD3 ratio of 0.38. After cell resting (Mon/CD3=0.05), detection rates increased significantly (CMV = 101, EBV = 78, ADV = 26; $p < 0.0001$). Following 5-year cryopreservation, despite cell resting, the median frequency of detectable VSTs remained reduced (CMV = 28, EBV = 5, ADV=2 per 300,000 cells).

Conclusion: Cell resting for 18–24 hours is essential for accurate VST quantification in CD45RA-depleted products. ELISpot testing is recommended before infusion of long-stored aliquots to ensure antiviral efficacy.

INTRODUCTION

Viral infections caused by cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (ADV) are among the leading infectious complications following hematopoietic stem cell transplantation (HSCT)^{1,2}. While antiviral drugs are widely used, their efficacy is often limited by drug resistance, toxicity, and delayed immune reconstitution.

Adoptive transfer of virus-specific T-lymphocytes (VSTs) has emerged as an effective therapeutic approach, capable of reconstituting immunity without exacerbating graft-versus-host disease (GVHD)³. The use of CD45RA-depleted T-cell products allows for the selective administration of memory T cells, reducing the risk of alloreactivity. The effectiveness of this approach has been demonstrated in numerous studies^{4,5}. At the Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, this type of therapy has been successfully applied for more than 10 years^{6,7}. However, a critical factor for the success of this strategy lies in the accurate evaluation of the frequency and function of VSTs in donor products^{8,9}.

This study aimed to assess the detectability of CMV-, EBV-, and ADV-specific T-lymphocytes in CD45RA-depleted donor cell products before and after cryopreservation, and to evaluate the impact of cell resting and long-term storage on VST detection.

Methodology

Peripheral blood samples were collected from 199 healthy donors. CD45RA-depleted fractions (167 samples) were obtained via immunomagnetic separation (CliniMACS, Miltenyi Biotec). These samples were divided into three groups: fresh CD45RA-depleted products (n=122), cryopreserved for 2–4 weeks (n=30), and cryopreserved for 5 years (n=15).

All donors of cell material in our Center sign such informed consent, which fully applies to the samples included in this study. Cryopreservation was performed using a programmable freezer (IceCube, Sy-lab®) at a cooling rate of 1°C/min with phase transition compensation. For cryopreservation, a 7.5% DMSO solution was used. Thawing was carried out in a 37°C water bath. Cell viability was assessed by flow cytometry (Beckman Coulter) using 7-AAD. Cell resting was performed in 6-well Corning plates (2×10⁶ cells/ml, 3 ml per well) in CTL Test Medium for 18–24 hours at 37°C.

VSTs were quantified using IFN-γ ELISPOT assay following in vitro stimulation with CMV pp65, EBV consensus, and AdV5 hexon peptide pools (all reagents were

from Biotec). All stimulators were used at a final concentration of 0.15 nmol/ml, as recommended by the manufacturer. The ELISPOT assay was performed according to the CTL ImmunoSpot reagent manufacturer's instructions. Briefly, 300,000 cells per well in a 96-well plate (200 μl volume) were incubated for 20–24 hours with stimulating peptides on Human IFN-γ Single-Color ELISPOT plates in CTL Test Medium. After incubation and spot development, the results were analyzed using a CTL S6 Flex M2 ELISPOT Reader. The final values were calculated after subtracting background activity from the negative control (spontaneous activation). Wells stimulated with the polyclonal T-lymphocyte activator phytohemagglutinin (PHA) served as a positive control. Test results were considered valid if negative control values were <10 spots and positive control values were >100 spots per 300,000 cells per well. Positive response thresholds were defined as >10 spots for CMV and EBV, and >4 spots for AdV per 300,000 mononuclear cells. The cutoff threshold for each pathogen was calculated as the maximum value observed in the negative control (spontaneous activation) + 3SD. The example of VST detection by ELISPOT assay is shown in **Figure 1**.

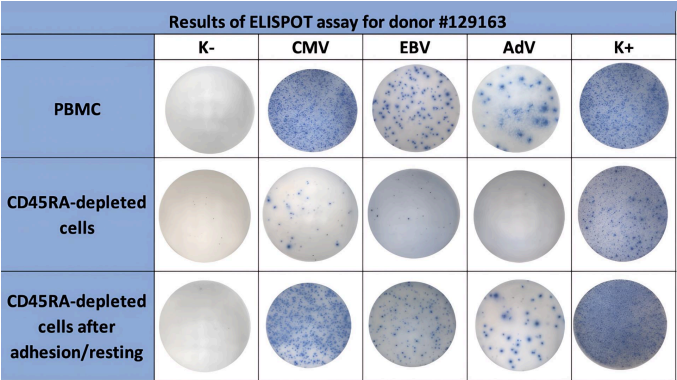


Fig. 1 Example of VST detection. Comparison of VST detectability in fresh samples from the same donor: PBMCs, CD45RA-depleted cells without resting, and CD45RA-depleted cells after 18–24 h resting. K- indicates negative control wells, and K+ indicates positive control wells.

Flow cytometry analysis was performed using CD3-APC (clone REA613/SK7), CD14-FITC (clone REA599), and 7-AAD (all reagents from Miltenyi Biotec). The Monocyte (CD14+ cells) to T-lymphocytes (CD3+ cells) ratio (Mon/CD3) was assessed before and after cell resting (BD FACS Cantoll). Statistical analysis was performed in RStudio Server 2024.09.1. Changes in quantities between different time points were assessed using the Mann-Whitney U-test for paired data. The level of statistical significance was set at 0.05.

Results

In peripheral blood samples from healthy donors (N=199), pathogen-specific T lymphocytes were readily detectable, with median values of 209 for CMV, 63 for EBV, and 34 for AdV. Cell viability across all groups remained consistently

high ranging between 80–95%. Thus, a sufficient pool of virus-specific cells could be expected in CD45RA-depleted products. Nevertheless, their detectability was sharply reduced after depletion, with median values of only 3, 1, and 0 for CMV, EBV, and AdV, respectively. The median Mon/CD3 ratio in peripheral blood MNCs was 0.12, whereas in fresh CD45RA-depleted fractions it increased to 0.38. After cell resting, Mon/CD3 ratio was reduced to 0.09 and Median detection rates of VSTs increased significantly: up to 101 for CMV-, 78 for EBV- and 26 for ADV-specific T-lymphocytes per 300,000 MNC ($p < 0.0001$), **Figure 2**.

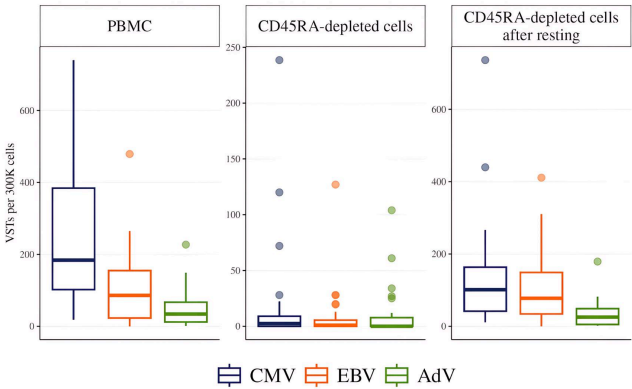


Fig. 2 Detection of VST at different stages of processing. The number of IFN- γ -producing cells per 300,000 MNCs was measured by ELISpot in (1) freshly isolated peripheral blood MNC ($N=199$), (2) CD45RA-depleted fractions ($N=122$), (3) CD45RA-depleted fractions after 18-24 hours of cell resting ($N=122$).

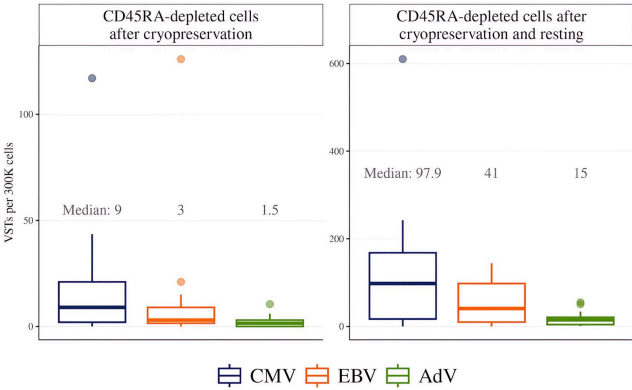


Fig. 3 Detection of virus-specific T cells after thawing and after subsequent cell resting. Shown are ELISpot results for CMV-, EBV-, and ADV-specific T cells per 300,000 mononuclear cells. Left panel: detection immediately after thawing ($N=30$). Right panel: detection after thawing with 18-24 hours of cell resting ($N=30$).

In CD45RA-depleted fractions cryopreserved for 2–4 weeks, VST detection was markedly reduced after thawing, with median values of 9, 3, and 1.5 for CMV, EBV, and AdV, respectively. Incorporation of an overnight cell resting step restored VST detectability, raising the medians to 98, 41, and 15, respectively (**Figure 3**).

Table 1 presents the Mon/CD3 ratio in CD45RA-depleted cell fractions at different stages of the experiment. Further detection of VST in CD45RA-depleted products was

carried out only after 18-24 hours of resting. Long-term storage (over 5 years) of cryopreserved CD45RA-depleted products significantly reduced VST activity compared to fresh samples and samples after short-term (2–4 weeks) storage. Long-term cryostorage (over 5 years) further diminished VST activity compared to both fresh samples and those stored for 2–4 weeks. Median values decreased from 101 to 28 for CMV, from 78 to 5 for EBV, and from 26 to 2 for AdV (**Figures 4–6**).

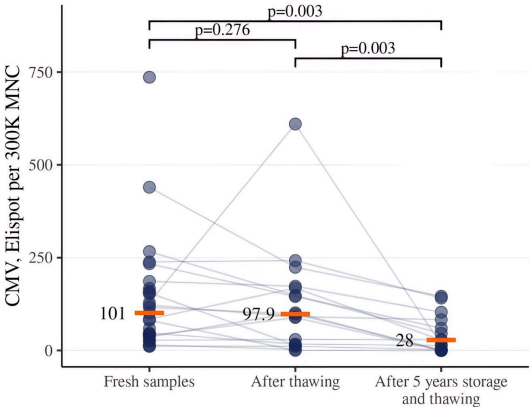


Fig. 4 Detection of CMV-specific T cells in CD45RA-depleted products after different storage conditions. Data is presented for fresh samples, cryopreserved samples stored for 2-4 weeks, and for samples stored for 5 years.

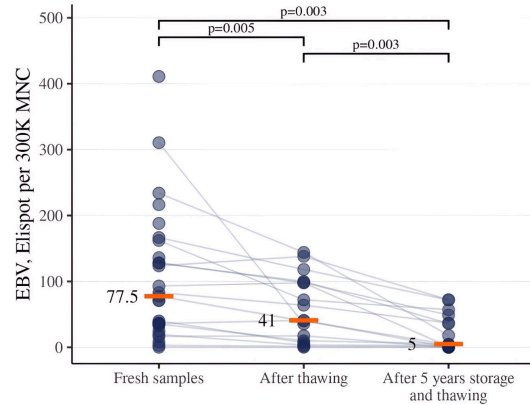


Fig. 5 Detection of EBV-specific T cells in CD45RA-depleted products after different storage conditions. Data is presented for fresh samples, cryopreserved samples stored for 2-4 weeks, and for samples stored for 5 years.

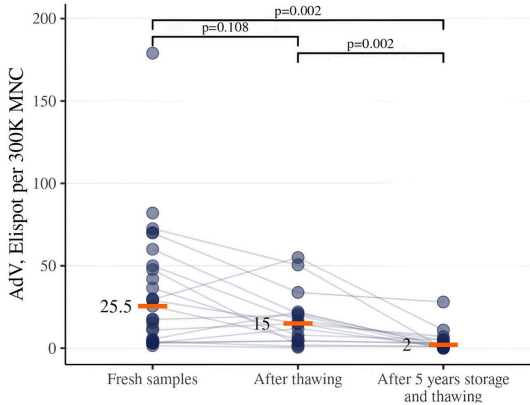


Fig. 6 Detection of AdV-specific T cells in CD45RA-depleted products after different storage conditions. Data is presented for fresh samples, cryopreserved samples stored for 2-4 weeks, and for samples stored for 5 years.

Table 1. Mon/CD3 ratio in CD45RA-depleted cell fractions at different stages of the experiment

Condition	N	Median (Q1–Q3)	Range (min–max)	p-value ¹	p-value (adjusted) ²
Fresh, before cryopreservation	27	0.38 (0.22–0.99)	0.07–3.92	<0.001	<0.001
Fresh, after 18–24h of resting	27	0.09 (0.05–0.15)	0.01–0.31		
After thawing (2–4 weeks), no resting	17	0.04 (0.02–0.06)	0.01–0.61	0.03	0.03
After thawing (2–4 weeks), after resting	17	0.03 (0.01–0.04)	0.01–0.17		
Fresh, after 18–24h of resting	15	0.02 (0.01–0.04)	0.01–0.04	N/A	N/A
After thawing + 18–24h resting (>5 years)	15	0.02 (0.01–0.04)	0.01–0.04		

Table shows Mon/CD3 ratios across different experimental conditions in CD45RA depleted products. Data were obtained by flow cytometry. Values are presented as medians with interquartile ranges (Q1–Q3), as well as minimum and maximum values. Statistical significance (p-value, adjusted p-value) was calculated for paired comparisons as indicated.

1. Mann–Whitney test for paired data

2. p-value adjusted for multiple comparisons using the Holm method

Discussion

It is well established that cryopreservation has a significant impact on the functional activity of blood cells^{10,11}. Our findings indicate that monocyte content influences ELISpot-based detection of VSTs in CD45RA-depleted products. However, beyond quantitative effects, functional alterations in monocytes may contribute to suppression of T-cell activation, as suggested by prior reports¹². Cell resting appears to restore both the cellular composition and the immunological state of monocytes, suggesting a bidirectional recovery process. Adding cell resting step to the ELISpot assay workflow is necessary for adequate determination of the content of pathogen-specific T-lymphocytes in CD45RA-depleted fractions for cell therapy¹³.

Despite functional decline due to cryopreservation, VSTs remain detectable even after 5 years of storage. These residual virus-specific cells could be utilized as ‘third-party’ donor material for further expansion, supporting the feasibility of VST manufacturing using long-term cryopreserved products¹⁴.

CD45RA-depleted products have long been applied in clinical practice and remain in demand among clinicians. In numerous cases, infusion of CD45RA-depleted donor lymphocyte infusions (DLI) is required at late time points after HSCT. In such scenarios, it is critical to evaluate both the quantity and functional state of memory T cells in the depleted product prior to clinical use^{15,16}. The present study outlines effective laboratory approaches for the detection of pathogen-specific T cells in samples intended for cellular therapy.

Conclusion

Cell resting for 18–24 hours is essential for accurate quantification of virus-specific T-lymphocytes in CD45RA-depleted donor products. Long-term cryopreservation significantly reduces VST functionality, although partial restoration is possible. ELISpot analysis is recommended prior to clinical use of thawed products.

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