

Toward Optimal Cryopreservation and Storage for Achievement of High Cell Recovery and Maintenance of Cell Viability and T Cell Functionality

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Cryopreservation of biological materials such as cells, tissues, and organs is a prevailing topic of high importance. It is employed not only in many research fields but also in the clinical area. Cryopreservation is of great importance for reproductive medicine and clinical studies, as well as for the development of vaccines. Peripheral blood mononuclear cells (PBMCs) are commonly used in vaccine research where comparable and reliable results between different research institutions and laboratories are of high importance. Whereas freezing and thawing processes are well studied, controlled, and standardized, storage conditions are often disregarded. To close this gap, we investigated the influence of suboptimal storage conditions during low-temperature storage on PBMC viability, recovery, and T cell functionality. For this purpose, PBMCs were isolated and exposed with help of a robotic system in a low-temperature environment from 0 up to 350 temperature fluctuation cycles in steps of 50 cycles to simulate storage conditions in large biorepositories with sample storage, removal, and sorting functions. After the simulation, the viability, recovery, and T cell functionality were analyzed to determine the number of temperature rises, which ultimately lead to significant cell damage. All studied parameters decreased with increasing number of temperature cycles. Sometimes after as little as only 50 temperature cycles, a significant effect was observed. These results are very important for all fields in which cell cryopreservation is employed, particularly for clinical and multicenter studies wherein the comparability and reproducibility of results play a crucial role. To obtain reliable results and to maintain the quality of the cells, not only the freezing and thawing processes but also the storage conditions should be controlled and standardized, and any deviations should be documented.

Keywords: PBMC, cryopreservation, temperature fluctuations, T-cell functionality, viability, recovery

Introduction

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) are commonly used not only in research of infectious diseases, such as HIV,^{1,2} malaria,³ and tuberculosis,⁴ but also in other fields of research such as cancer⁵ and dementia.^{6,7} As cells of the immune system, they play a crucial role in vaccine development^{8–10} and clinical trials.^{11–13} Therefore, large quantities of PBMC samples are often isolated and stored in biobanks. The storage in biorepositories enables multicenter^{10,14} as well as retrospective studies. Nowadays, a variety of samples such as blood, stem cells, tissues, oocytes, and sperms, as well as environmental specimens¹⁵ are stored in biorepositories. Optimal cryopreservation enables

storage of samples by maintaining viability and functionality.¹⁴ However, every sample reacts with different sensitivity to cryopreservation.

In the past, freezing and thawing processes have been studied extensively. Suboptimal freezing and thawing conditions lead to cell damage. There are two basic damage mechanisms. First, the mechanical damage caused by the formation of intracellular ice crystals,^{16,17} and second, the osmotic damages due to high intracellular salt concentrations as a result of water loss after crystallization. The loss of water has further consequences, which Farrant described as solution effects.¹⁸ Mazur et al. established the two-factor hypothesis wherein they described the optimal freezing rate for successful cryopreservation.¹⁹ Based on these findings,

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the freezing and thawing processes are now mostly standardized and controlled, for example through the use of freezing containers. To avoid biochemical reactions and recrystallization processes, the storage temperature should be below the glass transition temperature of -130°C .^{19–22}

However, in contrast to the freezing and thawing processes, the storage conditions are often much less controlled or standardized. Referring to this issue, some research groups studied the effect of storage time and temperature on cell viability and T cell functionality.^{23–28} The T cell response as an indicator of effective vaccines plays a crucial role in the development of current vaccines such as for HIV.²⁹ The maintenance of T cell functionality during cryopreservation is therefore very important to produce reliable results.^{10,26,30–34}

Uncontrolled storage can easily lead to multiple temperature changes during sample storage, sorting, and removal.³⁰ In 2013, Germann et al. studied the effect of short temperature rises during deep temperature storage to PBMC viability, recovery, and T cell functionality.³⁰ The cells were first exposed to 400 temperature cycles and then analyzed. They determined a reduction of all sampled parameters after 400 temperature shifts.³⁰

To complement this study, we isolated and froze PBMCs of different human donors and simulated suboptimal storage handling with a robotic system in a low-temperature environment. Therefore, we tried to mimic sample banking practice in a large biorepository. The basis of our hypothesis was that samples in biobanks are generally stored and removed several times a day. In this case, the samples, which are already stored in the biobank, are also involved in the new storage and removal processes and therefore also exposed to temperature rises. In contrast to Germann et al.³⁰ where the influence of temperature fluctuations on cell viability, recovery, and functionality was only determined after completing 400 cycles, we analyzed the effects after every 50th cycle step. This allows us to observe the minimum number of temperature cycles causing significant cell damage.

For this study, the isolated cells were frozen and exposed to 350 temperature fluctuation cycles. Three samples were removed after every 50th cycle step to analyze the influence of temperature fluctuations. PBMC viability and recovery were determined directly after thawing and after an overnight culture. The T cell functionality was quantified by interferon-gamma (IFN- γ) ELISpots.

Materials and Methods

Isolation of PBMCs

PBMCs were isolated from buffy coat samples from healthy cytomegalovirus (CMV)-seropositive donors at the blood donor center, Blutspendezentrale Saar-Pfalz gGmbH Am Klinikum Saarbruecken (Saarbruecken, Germany). They gave written informed consent that the buffy coats could be used for research purposes.

The isolation of PBMCs was performed by using density gradient centrifugation and Ficoll (Histopaque-1007; Sigma-Aldrich, Taufkirchen, Germany) as the PBMC separation medium. It separated the PBMCs from almost all erythrocytes, granulocytes, and dead cells. The PBMCs were collected and washed with phosphate-buffered saline (PBS; Gibco, Karlsruhe, Germany). The remaining erythrocytes were lysed. Then, 2×10^8 PBMCs were incubated in

Pharm Lyse (BD Biosciences, Heidelberg, Germany) for 30 minutes in the dark. PBMCs were purified by several washing steps.

Cryopreservation

Isolated PBMCs were frozen in cryomedium IBMT I (Fraunhofer IBMT, Sulzbach, Germany)³⁵ with a final concentration of 1×10^7 cells/mL in 1-mL aliquots. The samples were transferred into precooled ($+4^{\circ}\text{C}$), freezing isopropanol containers (Mr. Frosty™; Thermo Fisher Scientific, Dreieich, Germany) where the cells were frozen at a controlled cooling rate of $-1^{\circ}\text{C}/\text{min}$ from $+4^{\circ}\text{C}$ to -80°C . Twenty-four hours after the freezing process, the frozen cells were transferred into the vapor phase of liquid nitrogen (LN2) below -135°C for storage.

Simulation of suboptimal storage conditions

To simulate sample storage, sorting, and removal processes in biorepositories and clinical trials, PBMCs were cycled from below -130°C up to -60°C to 350 times in steps of 50 cycles (0, 50, 100, ..., 350) by a robotic system (Fig. 1). The samples were placed into a sample cabinet of the robotic arm. The robotic arm was able to change its vertical direction between two positions. In the first position, the samples were in the gas phase of LN2 below -130°C . By changing its vertical direction to the second position, the samples were exposed for about 5 minutes to room temperature until the temperature inside the samples reached a defined temperature of -60°C .

The temperature was measured with a Type T thermocouple embedded inside a reference sample. After warming to a defined temperature of -60°C , the samples were lowered to below -130°C in the vapor phase of LN2. The cooling and warming rates during the experiment correlate with the rates in a normal cryotank when samples are sorted and removed.

Every 50 cycles, three samples were replaced with dummies and transported, maintaining the cooling chain, into the gasphase of LN2 of a cryotank until further analysis. Thus, for every cycle condition (0, 50, 100, ..., 350), we had three samples per donor. The cycling process is described in detail in Figure 2.

Thawing PBMCs

The frozen PBMCs were thawed in a water bath at 37°C . One milliliter of prewarmed medium (RPMI 1648 medium [Gibco, Darmstadt, Germany] supplemented with 25 mM HEPES buffer [Gibco, Darmstadt, Germany], 1 mM L-glutamine [Gibco, Darmstadt, Germany], $1 \times$ penicillin/streptomycin [PAA; Cölbe, Germany], and 10% heat-inactivated fetal calf serum [FCS; [Gibco, Darmstadt, Germany]]) was added to the thawed cells. The cell suspension was transferred to 50-mL tubes (Greiner Bio-One, Frickenhausen, Germany) filled with 8 mL medium (end volume 10 mL). The cells were centrifuged at $400 \times g$ for 6 minutes at room temperature; 1×10^7 cells were resuspended in 10 mL medium and incubated overnight in a cell incubator at 37°C and 5% CO_2 .

Determination of cell viability and cell recovery

The influence of temperature changes during deep temperature storage on cell viability and cell recovery was

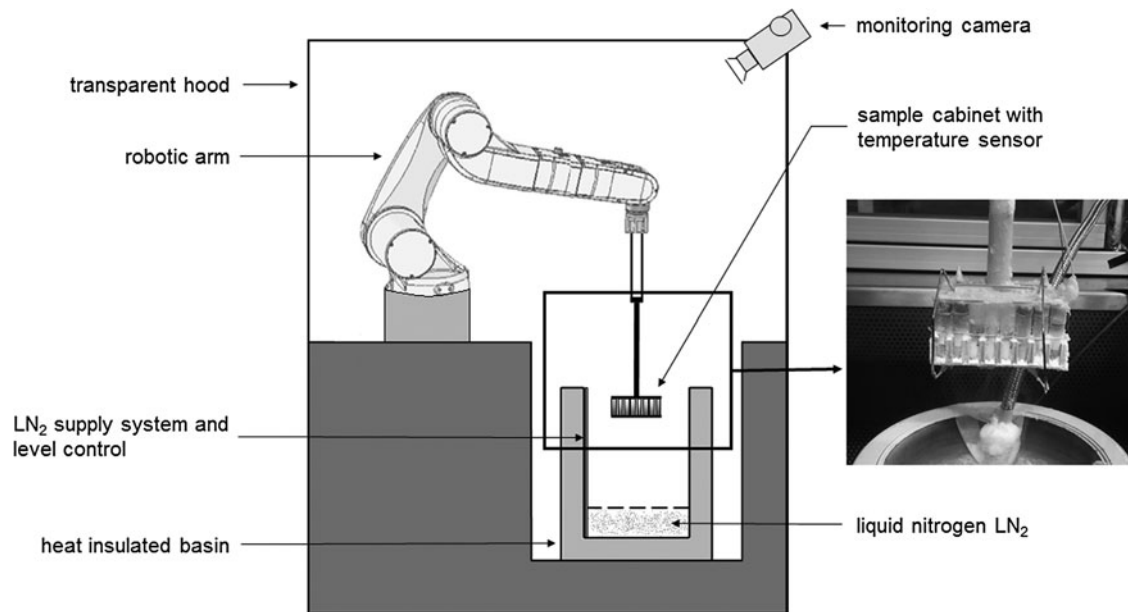


FIG. 1. Automated robotic system with robotic arm and sample cabinet. Robotic arm changed vertical position. Samples exposed to room temperature and temperature below -130°C . Temperature shift simulates storage conditions during biobanking. Modified from Germann et al.³⁰

determined by using the trypan blue dye exclusion test. The measurement was performed using an automated cell analyzer (ViCell cell analyzer; Beckman Coulter, Krefeld, Germany). The cell viability and cell recovery from three samples per cycle condition per donor were measured three times immediately after thawing as well as after overnight culture.

$$\% \text{viability} = \left(\frac{\text{number of viable PBMCs}}{\text{number of total PBMCs}} \right) \times 100$$

Viability:

Recovery (%) directly after thawing:

$$\% \text{recovery directly after thawing} = \left(\frac{\text{number of viable PBMCs after thawing}}{\text{number of frozen viable PBMCs}} \right) \times 100$$

Recovery (%) after overnight culture:

$\% \text{recovery after overnight culture} =$

$$\left[\frac{\text{number of viable PBMCs after overnight culture}}{\text{(number of frozen viable PBMCs} - \text{number of PBMCs removed for measurement directly after thawing)}} \right] \times 100$$

Determination of T cell functionality

IFN- γ ELISpots were performed to determine the influence of temperature changes during deep temperature storage on T cell functionality.

The immune response was stimulated by CMV pp65 peptide pool (cytomegalovirus; JTP, Berlin, Germany), CEF

peptide pool (peptide mixture of cytomegalovirus, Epstein-Barr virus, influenza virus; CTL, Aalen, Germany), and PHA (phytohemagglutinin; Thermo Fisher Scientific) as a positive control. Thawing medium containing 0.4% dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as background control.

After overnight culture, 1×10^5 PBMCs were added into the wells of a precoated anti-human-IFN- γ mAb 1-D1k 96-well plate (Mabtech AB, Hamburg, Germany). To stimulate an immune response, the different peptides were added to a final concentration of $2 \mu\text{g/mL}$ CMV, $4 \mu\text{g/mL}$ CEF, and $1 \mu\text{g/mL}$ PHA and incubated at 37°C and 5% CO_2 for 20–22 hours. After incubation, the plate was washed with PBS-Tween (PBS supplemented with 1% FCS and 250 μL Tween 20; Sigma-Aldrich). The production of IFN- γ was detected by using a biotinylated detection antibody (mAb 7-B6-1; Mabtech AB) diluted 1:500 in PBS +10% FCS and incubated for 3 hours and a streptavidin-horseradish peroxidase complex (Mabtech AB) diluted 1:300 in PBS +10% FCS and incubated for 1 hour. After the incubation time, the plate

was washed with PBS-Tween and PBS. The spot development was performed by using the Nova Red Substrate Kit (Vector, Burlingame, CA) and stopped after 15 minutes, washing the plate from every site with distilled water. After drying the plate overnight, the spots were evaluated with Immunospot Analyzer (CTL) and the results were specified as spot-forming cells (SFCs per million PBMCs).

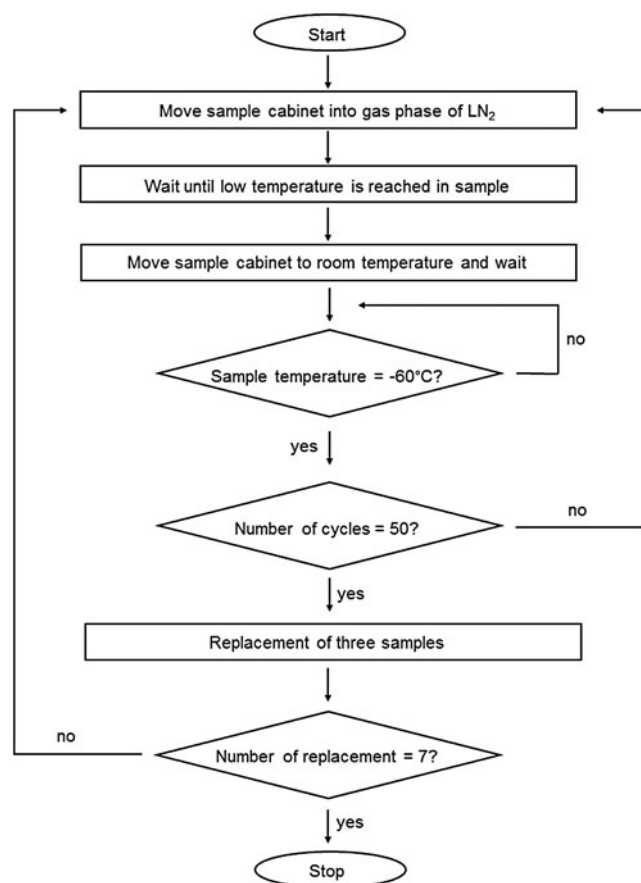


FIG. 2. Cycle program in detail. After starting the cycle program, the sample cabinet is moved into the cold gas phase above liquid nitrogen until a temperature below -130°C is reached in the reference sample. Next, the sample cabinet moves up into a room temperature zone until a defined temperature of -60°C is reached in the reference sample. This process is repeated until the last remaining samples are exposed to 350 cycles.

Quality assurance

The study was performed in a laboratory, which is under Good Clinical Laboratory Practice. There are standard operating procedures and the staff is well trained. The performance of ELISpot assay is a long-standing process in the laboratory and is periodically evaluated by an External Quality Assessment Program Oversight Laboratory (EQAPOL) and ELISpot Quality Assurance Program.

Statistics

Statistical tests were performed using OriginPro 9.0. All data are shown as mean \pm standard deviation. For statistical testing, we used the individual values. In summary, for every cycle condition, we performed 36 individual measurements for viability, 36 for recovery, and triplicates for T cell functionality. After testing the sample population for normal distribution through the Kolmogorov-Smirnov test, statistical differences between storage with and without temperature fluctuations were calculated by using a one-way analysis of variance (ANOVA) Bonferroni test. To quantify the reduction of T cell functionality, a linear regression

analysis over the numbers of cycles was performed. A p -value of 0.05 is specified as the significance level.

Results

Influence of temperature cycles on PBMC viability and recovery

To study the influence of temperature changes during deep temperature storage, PBMCs from four CMV-positive donors were isolated and exposed to 0–350 temperature fluctuations in steps of 50 cycles with a robotic system. After the simulation of temperature fluctuations during cell storage, the PBMC viability and recovery were determined immediately after thawing as well as after overnight culture, through a trypan blue exclusion test.

The viability of the samples stored without temperature fluctuations in the vapor phase of LN₂ directly after thawing was $98.33\% \pm 0.65\%$ (Fig. 3A). After 350 temperature cycles, the viability decreased to $94.03\% \pm 5.65\%$. The difference became statistically significant after 200 temperature fluctuations ($N=36$, one-way ANOVA Bonferroni with $p < 0.05$, $R^2 = 0.17051$, $\eta^2 = 1.0089$). After overnight culture, the viability of the samples stored in the gas phase of LN₂ was $95.35\% \pm 1.63\%$ and decreased to $93.87\% \pm 1.83\%$ after 350 temperature cycles (Fig. 3B). A significant difference was observed after 200, 250, and 350 temperature cycles ($N=36$, one-way ANOVA Bonferroni with $p < 0.05$, $R^2 = 0.06666$, $\eta^2 = 1.0019$).

Like the PBMC viability, the recovery decreased with increasing numbers of temperature fluctuations, but to a higher degree. After thawing, the recovery was $96.08\% \pm 6.91\%$ (0 cycles) and decreased to $74.86\% \pm 11.97\%$ after 350 cycles (Fig. 4A). A significant difference, after thawing, occurred after 150 temperature cycles (Fig. 4A; $N=36$, one-way ANOVA Bonferroni with $p < 0.05$, $R^2 = 0.44609$, $\eta^2 = 1.0096$). After an overnight rest, the recovery of samples stored in the gas phase of LN₂ was $81.41\% \pm 6.20\%$ and decreased to $57.74\% \pm 9.94\%$ after 350 cycles (Fig. 4B). After overnight culture, a significant difference was observed after only 50 temperature fluctuations (Fig. 4B, $N=36$, one-way ANOVA Bonferroni with $p < 0.05$, $R^2 = 0.502$, $\eta^2 = 1.0076$).

Influence of temperature cycles on T cell functionality

To examine a possible influence of temperature fluctuations on the T cell functionality, IFN- γ ELISpots were performed. To specify positive T cell responses, the average number of SFCs/ 1×10^6 PBMCs was determined from three replicate wells. Following standardization and validation issues of ELISpot assay,³⁶ we used the definition of responder $R > 4D$ and $R > 55$, where R is the SFCs/ 1×10^6 PBMCs for the reagents (CEF and CMV) and D corresponds to SFCs for background. All samples were positive for both CEF and CMV. Furthermore, we classified the immune response of donors in IFN- γ ELISpot in four different groups to compare the reactivity: negative < 55 SFCs/ 1×10^6 PBMCs, low responder < 600 SFCs/ 1×10^6 PBMCs, medium responder < 2000 SFCs/ 1×10^6 PBMCs, and high responder > 2000 SFCs/ 1×10^6 PBMCs.

After CEF stimulation, none of four donors were classified negative, two donors as low responders, one donor as

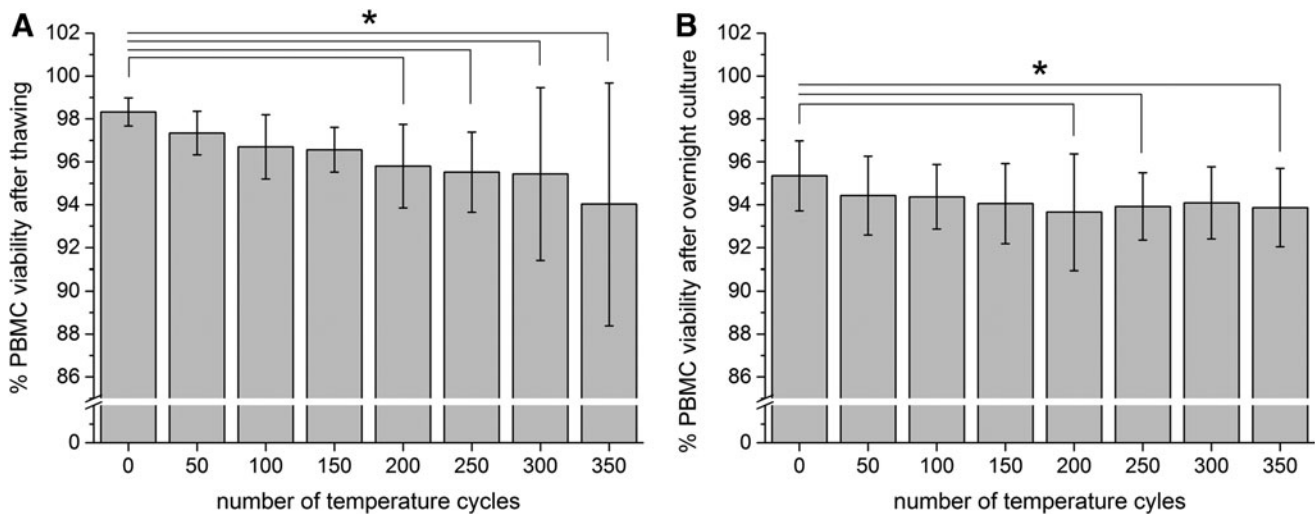


FIG. 3. PBMC viability after thawing (A) and after overnight culture (B). Figures show the PBMC viability after 0–350 temperature cycles. PBMC viability determined directly after thawing and after overnight culture. Figures show mean \pm standard deviation. *Statistical difference to 0 cycles with $p < 0.05$. PBMC, peripheral blood mononuclear cell.

medium responder, and one donor was classified as high responder (Table 1). After stimulation with CMV, none of four donors were classified negative, two donors as low responders, and two donors as high responders (Table 2).

Donor 1 showed a high response on CEF as well as on CMV stimulation. Except for 350 cycles, the T cell response decreased with increasing numbers of temperature cycles (Tables 1 and 2). For Donor 2, a low responder, a decrease with increasing numbers of temperature cycles after both CEF and CMV stimulation was also observed. Donors 3, apart from 350 cycles, and 4 showed similar behavior (Tables 1, 2). After stimulation with CEF, the decrease of the high responder cells became significant after 250 and 300 cycles and medium responder's cells after 200 cycles. The low responder cells showed no significant degradation.

After stimulation with CMV peptide pool, the decrease in high responder cells of Donor 1 was significant after 200,

250, and 300 temperature cycles and for Donor 3 after 100 cycles. The low responder cells showed no statistically significant decrease.

To present the decrease in a clearer manner, we pooled and normalized the data of all donors. A linear regression model was used to calculate the relative reduction per 50 cycles in percent (%). After stimulation with either CEF or CMV peptides, the immune response decreased with increasing numbers of temperature fluctuations. After 50 cycles, the T cell response decreased about $-11.39\% \pm 9.52\%$ after CEF stimulation and about $-7.38\% \pm 8.73\%$ (Fig. 5A). The decrease reached a maximum of $-27.70\% \pm 13.80\%$ after CEF stimulation (Fig. 5A) and $-22.52\% \pm 11.44\%$ after CMV stimulation after 350 temperature cycles (Fig. 5B).

The pooled data of each 50th cycle group show no significant differences ($N=12$, one-way ANOVA Bonferroni with $p < 0.05$, R^2 [CEF]=0.50355, R^2 [CMV]=0.36517).

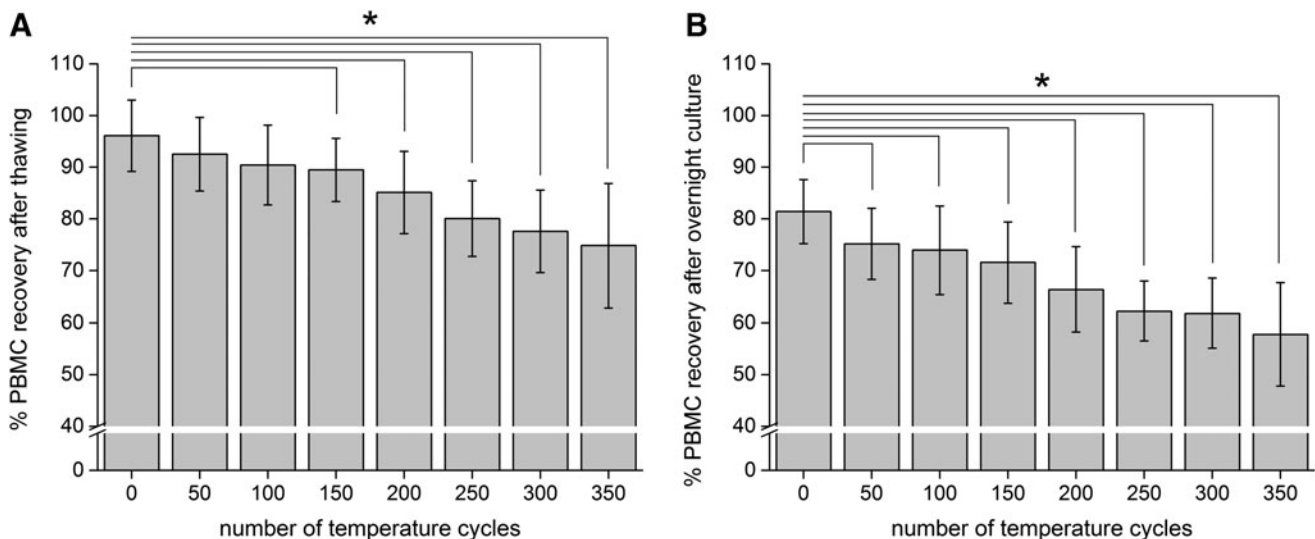


FIG. 4. PBMC recovery after thawing (A) and after overnight culture (B). Figures show the PBMC recovery after 0–350 temperature cycles. PBMC recovery determined directly after thawing and after overnight culture. Figures show mean \pm standard deviation. *Statistical difference to 0 cycles with $p < 0.05$.

TABLE 1. INTERFERON-GAMMA ELISPOT RESPONSE OF FOUR DIFFERENT CMV-SEROPOSITIVE DONORS STIMULATED WITH CEF PEPTIDE POOL AFTER TEMPERATURE CYCLES

No. of temperature cycles	Donor 1, high responder	Donor 2, low responder	Donor 3, medium responder	Donor 4, low responder
CEF				
0	4120 ± 365	367 ± 6	1440 ± 269	563 ± 32
50	4223 ± 180	317 ± 90	1217 ± 99	457 ± 71
100	4007 ± 142	320 ± 17	1170 ± 87	570 ± 123
150	3867 ± 55	430 ± 26	1363 ± 0	400 ± 46
200	3660 ± 200	323 ± 70	897 ± 126	417 ± 121
250	3460 ± 105	310 ± 115	810 ± 92	410 ± 62
300	3427 ± 164	267 ± 38	790 ± 30	380 ± 36
350	3797 ± 23	260 ± 50	930 ± 168	347 ± 38

Values show mean ± standard deviation of SFCs per 1×10^6 PBMCs from three replicate wells.

PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells.

The linear regression model yielded a significant reduction of the gradient of $-3.88\% \pm 0.82\%$ ($N=7$, $R^2=0.81607$, $p=0.00529$) after every 50th cycle step, after stimulation with CEF peptides, and $-2.86\% \pm 0.78\%$ ($N=7$, $R^2=0.72575$, $p=0.01494$) after every 50th cycle step after stimulation with CMV peptides.

Discussion

Cryopreservation below -130°C is widely used in clinical trials,^{11,12} multicenter studies,¹⁰ and in almost every biological and biomedical research field. It enables long-term storage,^{24,31} maintaining functionality and viability. A variety of samples such as stem cells, blood, oocytes, and sperms, or environmental samples are stored in large amounts in biorepositories. While the freezing and thawing processes are well standardized, the storage conditions are often less controlled. Several research groups showed that suboptimal storage conditions lead to cell damage.^{26,30,37} Because cryopreservation is becoming increasingly impor-

tant, our findings are of great interest, particularly for biobanks and clinical trials.

In this study, we showed that a small number of temperature rises from below -130°C up to -60°C during deep temperature storage negatively affects the viability, recovery, and T cell functionality of PBMCs. We further detected the number of such temperature rises, which leads to significant cell damages.

For this purpose, we exposed isolated and frozen PBMCs from four different donors from below -130°C up to -60°C , from 0 to 350 temperature cycles, to mimic storage conditions within sample sorting, removal, and transfer in biobanking and clinical trials. In this process, the samples are not thawed, only reaching a temperature of -60°C . This temperature was chosen because it is a critical temperature where recrystallization processes and biochemical reactions can occur. The incorporation of PBMCs from different donors in this study was chosen to get a first qualitative insight into interperson variability.

However, the question of this study was especially if we can observe the same general trend for all donors. During the simulation of suboptimal storage conditions, we removed samples after every 50th cycle step to determine the influence on PBMC viability and recovery by the trypan blue exclusion test and on T cell functionality by means of IFN- γ ELISpot. The assays and methods in this study show the expected variability when working with cell suspensions, such as potential cell agglomeration, counting errors, transfer losses, and other sources of variability.

We observed high viability and recovery in our control cells continuously stored below -130°C in the gas phase of LN2, demonstrating the suitability of our freezing and thawing processes. Similar control results were reported by some other research groups.^{24,32,38} This indicates that optimized cell cryopreservation and storage conditions without temperature fluctuations lead to a suitable recovery and maintenance of cell viability and functionality for subsequent immune assays.

We further found that all examined parameters, the viability, the recovery, and the T cell functionality, decreased nearly proportionally with increasing numbers of temperature cycles during deep temperature storage. Obviously, any processes, which lead to cell damages, occur during the warming process from -130°C up to -60°C , for example, biochemical reactions and ice recrystallization processes above the glass transition temperature. They can damage the cell membrane and the cytoskeleton in multiple ways.^{16,17,21} It is also known that during the freezing and thawing processes, two main cell damage mechanisms take place, which are described by Lovelock.³⁹⁻⁴² On the one hand, this is due to the formation of intracellular ice crystals,¹⁷ and on the other hand, due to osmotic damages, which are a result of high salt concentration due to water loss during crystallization. The water loss has additional consequences, which are also described as solution effects.¹⁸ The phospholipid membrane can be influenced by dehydration of the cell, leading to an increased sensitivity to mechanical effects.^{43,44} Furthermore, the tertiary structure can be damaged due to the high intracellular salt concentration, which can lead to loss of functionality, including denaturation of proteins. Accordingly, we suspect that these processes also took place during the warming process from below -130°C up to -60°C .

TABLE 2. INTERFERON-GAMMA ELISPOT RESPONSE OF FOUR DIFFERENT CMV-SEROPOSITIVE DONORS STIMULATED WITH CMV PEPTIDE POOL AFTER TEMPERATURE CYCLES

No. of temperature cycles	Donor 1, high responder	Donor 2, low responder	Donor 3, high responder	Donor 4, low responder
CMV				
0	4863 ± 195	187 ± 42	6280 ± 140	237 ± 38
50	4993 ± 133	170 ± 36	5970 ± 125	193 ± 32
100	4807 ± 182	210 ± 50	5807 ± 29	140 ± 35
150	4620 ± 10	170 ± 20	5960 ± 118	200 ± 20
200	4297 ± 272	137 ± 31	5130 ± 159	167 ± 45
250	4073 ± 59	197 ± 81	5697 ± 172	170 ± 20
300	3827 ± 120	153 ± 25	5363 ± 25	177 ± 46
350	4500 ± 108	130 ± 10	5033 ± 171	160 ± 70

Values show mean ± standard deviation of SFCs per 1×10^6 PBMCs from three replicate wells. SFC, spot-forming cell.

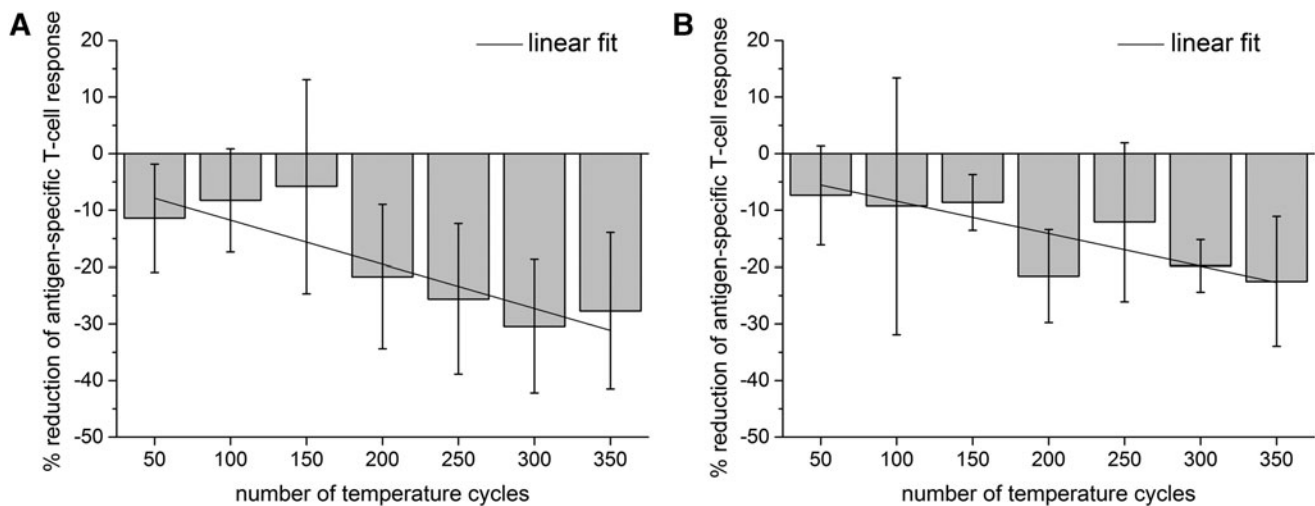


FIG. 5. Reduction of antigen-specific T cell response. (A) Shows reduction of T cell response after stimulation with CEF peptide pool and (B) after stimulation with CMV peptide pool. To show the reduction in a clearer way, a linear fit was performed and is presented as a *line*. Figures show reduction of immune response after 50–350 temperature cycles in comparison with 0 cycles. Figures show mean \pm standard deviation of four donors. The gradients are significantly different from zero temperature cycles with $p < 0.05$.

We observed a decrease of recovery and viability after overnight culture in comparison with the measurement directly after thawing. This may not only be due to normal apoptosis and necrosis processes during overnight culture,^{25,29,45,46} but it may also be caused by preapoptotic cells, which are triggered by cryopreservation to induce apoptosis during overnight culture.²³ This difference did not change with increasing numbers of cycles (data not shown). Moreover, Smith et al. showed by simulating transport conditions that the number of apoptotic cells increases and additionally the T cell response decreases.²⁶

The T cell response is a crucial factor in vaccine development.²⁹ Therefore, reliable performance of the ELISpot assay is important. Thus, we also investigated the influence of suboptimal storage on T cell functionality. We observed a high T cell response for the control cells stored below -130°C without temperature fluctuations. Furthermore, we observed that the T cell response of the cells, which were exposed to the temperature rises, decreased with increasing numbers of temperature cycles. Due to the expected variability when working with cell suspensions together with the small intervals of 50 temperature cycles, not every interval data group significantly differs from the one before or after.

Nevertheless, the linear regression analysis, statistically and graphically, shows a significant reduction of the antigen-specific T cell response over increasing number of temperature cycles. We suspect that changes of cell surface molecules and changes of expression levels, which were described by Costantini et al. (2003), are also triggered by the temperature rises from below -130°C up to -60°C .⁴⁷ Furthermore, Kreher et al. showed that cytokine secretion changed due to freezing and thawing processes.³⁸ This could explain the observed reduction in T cell response in ELISpot.

Above all, the maintenance of T cell functionality during cryopreservation is controversially discussed. Regarding the T cell immune response, there are different observations. Meanwhile, Owen et al. noticed the loss of T cell response, and Kreher et al. observed the maintenance of antigen-specific T cell response.^{25,38}

We suspect that the storage conditions may potentially be responsible for the different observations. Thus, for optimal storage conditions, it is important to avoid temperature fluctuations. The detection and documentation of deviations in deep temperature storage play a crucial role, especially in terms of storage duration and sample history. Samples, which are often stored for several years, could potentially degrade with regard to various examined parameters. Moreover, sample history regarding temperature fluctuations in long-term storage is most often unknown. To understand this retrospectively, the temperature should be recorded over longer periods in regular short intervals. It would be an improvement toward standardization and optimal cryopreservation if updated quality management systems and standard operating procedures would be established.

Although the cell suspension did not thaw completely, but merely warmed up to -60°C , the temperature rises sufficed to lead to multiple cell damages. In summary, we showed that temperature rises during deep temperature storage lead to reduction of viability, recovery, and T cell functionality of PBMCs. The study emphasizes the need for controlled storage, especially in clinical studies and biobanks, to yield comparable and reliable results and to maintain the quality of cryopreserved samples.

Furthermore, to guarantee controlled storage and handling without interruption of the cooling chain, protective hood systems, wherein the working area for sample removal and sorting is cooled down, are recommended. This would reduce the temperature fluctuations to a minimum. Moreover, establishment of an updated quality management system and introduction of standard operating procedures wherein, among others, deviations are documented would lead to a better standardization and quality of the respective samples.

In the future, more attention should be paid to the storage conditions to achieve a high recovery, maintain the viability, and the T cell functionality of cells. Moreover, studies are needed to determine whether other cell types such as stem cells and other biological materials react in a similar way to temperature cycles during cryostorage. In addition, further

aspects, including protein degradation, will be included in subsequent studies.

Acknowledgments

The authors thank B. Kemp-Kamke for her excellent technical assistance. This work was supported by the Bill & Melinda Gates Foundation grant number OPP38580_01.

Author Disclosure Statement

No conflicting financial interests exist.

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