ADAPTING THE PROTOCOL FOR STUDYING THE FUNCTIONAL CAPACITY OF T LYMPHOCYTES THAWED FROM CRYOPRESERVATION

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ABSTRACT

Background. *Immunological studies are impossible without long-term storage of cryopreserved biomaterial. There are no standard procedures for working with cryopreserved mononuclear leukocytes.*

The aim of the study. *To optimize the protocol for culturing T lymphocytes thawed after cryopreservation by assessing their viability and proliferative capacity.*

Methods. *Mononuclear leukocytes were isolated from the peripheral blood of relatively healthy volunteers (n = 18). Cells were subjected to controlled freezing down to –80 °C and were transferred to liquid nitrogen. First step: after thawing, the cells were stained with CFSE (carboxyfluorescein succinimidyl ester), were divided into two parts and cultured in the presence/absence of interleukin 2 (IL-2). Cell proliferation was stimulated with phytohemagglutinin (type P). Cells were incubated for 7 days. Sample analysis was performed using flow cytometry. Second stage: thawed cells were divided into three parts. Two parts were resuspended in a full growth medium with IL-2 and were placed in a thermostat (+37 °C) to "rest" for one hour or overnight. After "resting", the cells were stained with CFSE. One third of the thawed leukocytes were stained with CFSE immediately after thawing. Cells were stimulated, cultured and analyzed the same way at both stages of the study.*

Results. *It has been established that adding IL-2 to the culture medium contributes to a better cell survival. In the presence of IL-2, stimulated $CD4^+$ and $CD8^+$ T lymphocytes produced more daughter cell generations. At the end of the 7-day incubation "rested" samples had reduced leukocyte counts compared to the samples that were cultured immediately after thawing. The number of daughter cell generations formed by stimulated $CD4^+$ and $CD8^+$ T cells decreased when the "rest" stage was included into the study protocol.*

Conclusion. *Adding IL-2 into culture medium can increase the viability and mitotic capacity of thawed T cells, making their state more similar to that of freshly isolated lymphocytes. Cell "rest" after thawing negatively affects the viability and proliferative activity of T lymphocytes during their weekly incubation.*

Key words: *T lymphocytes, cryopreservation, primary cell culture, interleukin 2, rest*

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МОДИФИКАЦИЯ ПРОТОКОЛА ИССЛЕДОВАНИЯ ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ ОТТАЯВШИХ ПОСЛЕ КРИОКОНСЕРВАЦИИ Т-ЛИМФОЦИТОВ

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РЕЗЮМЕ

Обоснование. *Иммунологические исследования невозможны без длительного хранения биоматериала в условиях криоконсервации. Стандартные методики работы с мононуклеарными лейкоцитами, подвергавшимися криоконсервации, отсутствуют.*

Цель исследования. *Оптимизировать протокол культивирования оттаявших после криоконсервации Т-лимфоцитов по оценке их жизнеспособности и пролиферативной активности.*

Методы. *Мононуклеарные лейкоциты выделяли из периферической крови относительно здоровых добровольцев (n = 18). Клетки подвергали контролируемому замораживанию до –80 °С и переносили в жидкий азот. Первый этап: после оттаивания клетки окрашивали CFSE (carboxyfluorescein succinimidyl ester), делили на две части и культивировали в присутствии/отсутствии интерлейкина 2 (ИЛ-2). Пролиферацию клеток стимулировали фитогемагглютинином-П. Клетки инкубировали в течение 7 суток. Анализ образцов проводили методом проточной цитофлюориметрии. Второй этап: оттаявшие клетки делили на три части. Две части ресуспендировали в полной питательной среде с ИЛ-2 и помещали в термостат (+37 °С) для «отдыха» на 1 час или на ночь. После «отдыха» клетки окрашивали CFSE. Третью часть размороженных лейкоцитов окрашивали CFSE сразу после оттаивания. Клетки стимулировали, культивировали и анализировали единообразно на обоих этапах исследования.*

Результаты. *Установлено, что добавление ИЛ-2 в культуральную среду способствует лучшему выживанию клеток. Кроме того, в присутствии ИЛ-2 стимулированные $CD4^+$ и $CD8^+$ Т-лимфоциты производят больше дочерних генераций. По сравнению с пробами, сразу помещёнными в культуру, в пробах, прошедших «отдых», снижено число лейкоцитов по окончании 7-суточной инкубации. Количество дочерних генераций, формируемых стимулированными $CD4^+$ и $CD8^+$ Т-клетками, снижается при включении этапа «отдых» в протокол исследования.*

Заключение. *Внесение ИЛ-2 в культуральную среду может увеличить жизнеспособность и митотическую активность размороженных Т-клеток, приближая их состояние к таковому свежевыделенных лимфоцитов. «Отдых» клеток после оттаивания оказывает негативный эффект на жизнеспособность и пролиферативную активность Т-лимфоцитов при их последующей недельной инкубации.*

Ключевые слова: *Т-лимфоциты, криоконсервация, культивирование, ИЛ-2, отдых*

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BACKGROUND

Effective research in the field of immunology is impossible without constant access to biological material, which requires its collection, accumulation and storage. Today, the only way to store peripheral blood nuclear cells for a long time is to cryopreserve them: keep them at very low temperatures (–80…–196 °C). The use of biobanks, which allow biological samples to be accumulated and stored in liquid nitrogen, significantly facilitates research aimed at studying the causes of various diseases, developing and testing drugs [1].

It should be noted that cryopreservation can affect the expression of phenotypic markers and the functional activity of peripheral blood mononuclear cells [2, 3]. To mitigate the cryopreservation effects, a number of researchers [4, 5] proposed to introduce a "rest" stage into the work protocol after the thawing procedure: placing the cells in a complete culture medium (CCM) for a period of 1 to 24 hours at a temperature of +37 °C. It was shown that this procedure has a positive effect on the cells, in particular, it promotes the functionality restoration of antigen-specific T lymphocytes when assessed by the ELISPOT method [6–10]. Other researchers [11–14] proposed enriching the culture medium with additives such as cytokines, in particular interleukin 2 (IL-2), sodium pyruvate, nonessential amino acids, β-mercaptoethanol, etc., which should promote better survival of T lymphocytes in culture and ultimately increase their functional activity. However, to date, methods for working with mononuclear leukocytes that have undergone long-term (more than 24 months) cryopreservation have not been developed.

THE AIM OF THE STUDY

To optimize the protocol for culturing T lymphocytes thawed after cryopreservation by assessing their viability and proliferative capacity.

METHODS

Study participants. Volunteers (n = 18; 39 % women; average age 37.4 ± 1.2 years) participated in the study as peripheral blood donors. The inclusion criterion for the study was age over 18 years. Exclusion criteria: acute infectious diseases less than 4 weeks before the start of the study; pregnancy.

Obtaining biomaterial. Blood was collected on an empty stomach from the cubital vein into vacuum tubes (Weihai Hongyu Medical Devices Co, Ltd., China) containing ethylenediaminetetraacetic acid. Mononuclear leukocytes were isolated by the standard method by centrifuging twice diluted blood with Dulbecco's phosphate-buffered saline (DPBS; Gibco, USA) in a Diacoll density gradient (1.077 g/ml; Diaem, Russia). Isolated cells were washed twice in DPBS solution and placed in a medium containing 90 % heat-inactivated fetal calf serum (FCS; Biowest, Columbia) and 10 % intracellular cryoprotectant dimethyl sulfoxide (AppliChem, Germany). Cells resuspended in this medium were transferred to cryovials and subjected to controlled freezing in commercial CoolCell racks (Corning, USA) with a controlled temperature decrease rate (–1 °C/min) in a freezer (–80 °C) for 24 hours to minimize cell damage. Samples were then transferred to a liquid nitrogen tank (–196 °C) and stored until further use. The average storage time of samples was 40 ± 1.4 months.

Before the study, mononuclear leukocytes were thawed at +37 °C in a water bath for 1–2 min. The cells were transferred to 15 ml tubes, after which 10 ml of CCM were added dropwise to the samples: RPMI-1640, containing 25 mM Hepes and 2 mM L-glutamine (Gibco, USA), with the addition of 10 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, USA). The samples were gently mixed, tilted from side to side, and centrifuged for 10 min at 400 g. The cell pellet was resuspended in CCM. The viability of thawed leukocytes, when assessed with the standard trypan blue method, was at least 92 %.

Cultivation of mononuclear leukocytes. In the first stage of the study, the cells prepared after thawing were stained with 5 μM 5,6-carboxyfluorescein diacetate-N-succinimidyl ester (CFSE, carboxyfluorescein succinimidyl ester; Biolegend, USA) and washed twice with RPMI-1640 medium containing 20 % FCS. The cells were then counted in a Goryaev chamber, divided into two parts and resuspended at a concentration of 1×10^6/ml in CCM with or without IL-2 addition (100 ng/ml; Gibco, USA) (fig. 1a). Cell proliferation was stimulated with phytohemagglutinin-P (PHA; Serva, Germany) at a final concentration of 15 μg/ml. Unstimulated cells were used as a control. The total duration of leukocyte cultivation was 7 days, with the culture medium in each sample being replaced with a medium of similar composition on the 3rd–4th days. To maintain a constant pH of the medium, the cells were incubated in a desiccator with a candle placed in a thermostat (+37 °C). At the end of the incubation time, the cells were collected, counted, and stained with anti-CD3-BV605, anti-CD4-PE, and anti-CD8-BV510 antibodies (Biolegend, USA). Zombie UV dye (Biolegend, USA) was used to assess the viability of mononuclear leukocytes.

In the second stage of the study (fig. 1b), the cells were cultured in CCM containing 100 ng/ml IL-2. The mononuclear leukocytes obtained after thawing were divided into three parts, two of which were placed in the culture medium and put into a thermostat (+37 °C) for "rest" for 1 hour (H) or overnight (O). After "resting", the cells were stained with CFSE, stimulated with PHA, and cultured according to the previously described protocol. The third part of the thawed leukocytes was stained with CFSE immediately after thawing (I) and cultured in the presence of PHA according to the above protocol. Cultivation, medium change, cell counting, and analysis of the results were carried out uniformly throughout the study.

Flow cytometry. Analysis of mononuclear leukocytes was performed using a CytoFLEX S flow cytometer (Beckman Coulter, USA). The gating approach is shown in Figure 2.

Statistical data processing. Statistical analysis and data visualization were performed using GraphPad Prism 8 software (GraphPad Software, USA). Quantitative data in the text and tables are presented as means and their standard errors. Student's *t*-test was used to compare two groups of quantitative data; one-way ANOVA was used for several groups; multiple comparisons between groups were performed using Tukey's test. The critical significance level for testing statistical hypotheses was taken to be 0.05.

RESULTS

Modification of complete culture medium by introducing IL-2. The viability of peripheral blood mononuclear leukocytes thawed after long-term (40 ± 1.4 months) cryopreservation and cultured for 7 days under various conditions was analyzed. It was shown that the number of cells sharply decreases at the end of the cultivation period: on average, 33 % of the number of initially introduced cells remained in the sample.

At the same time, it was found that the IL-2 addition to the culture medium promotes better survival of mononuclear leukocytes (fig. 3). The most pronounced positive effect on viability was noted among cells stimulated with PHA. Thus, the number of leukocytes in samples containing IL-2 was significantly higher than in samples without added cytokine ($p < 0.001$). Similar results were obtained in the study of unstimulated cells. However, under these cultivation conditions, the differences in the number of cells between samples containing and not containing IL-2 did not reach the level of statistical significance ($p > 0.05$).

A positive effect of exogenously added IL-2 on the proliferative capacity of T cells thawed after long-term cryopreservation was revealed. It was shown that in the presence of this cytokine *in vitro* stimulated $CD4^+$ and $CD8^+$ T lymphocytes produce more daughter generations (fig. 4).

Thus, it was determined that the IL-2 addition to the culture medium increases both the viability and the proliferative capacity of T lymphocytes that have undergone long-term cryopreservation.

The mononuclear leukocyte thawing protocol modification by introducing a cell "rest" stage. Three cell`s thawing protocols were studied: without "rest", with an hour or overnight "rest". In each case, the culture medium contained IL-2.

A negative effect of "rest" on the viability of mononuclear leukocytes in the culture was established. Cell counting after 7 days of incubation with PHA showed that, compared to samples "I", the number of leukocytes in samples "H" ($p < 0.05$) and "O" ($p < 0.001$) was reduced

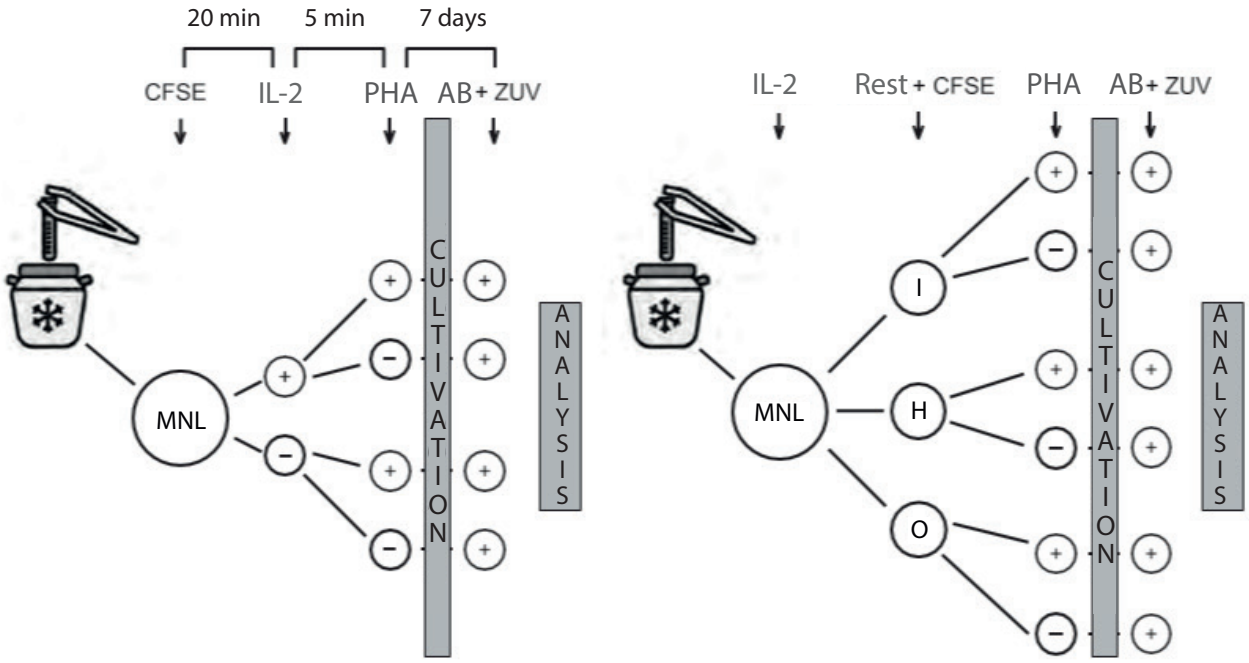

FIG. 1.
Study overview: a – first stage of the experiment; b – second stage of the experiment. MNL – mononuclear leukocytes; AB + ZUV – a cocktail of anti-CD3-BV605, anti-CD4-PE, anti-CD8-BV510 antibodies and Zombie UV vital stain. Types of "rest": I – immediately after thawing (without "rest"); H – hourly "rest"; O – overnight "rest"

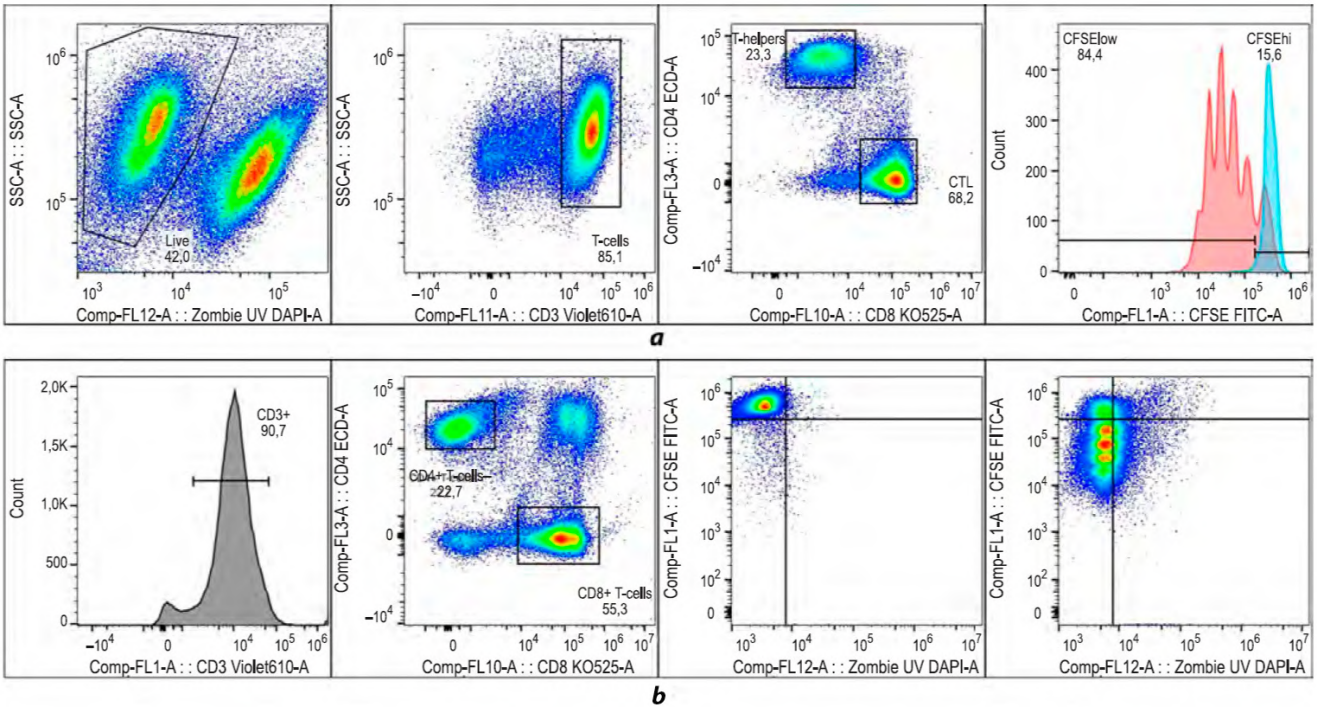

FIG. 2.
The gating approach used to distinguish mononuclear leukocytes (typical scatter diagrams). a – enumeration of daughter cell generations in stimulated T lymphocyte samples: viable elements isolation followed by CD3+ T cells gating, subsequent CD4⁺ and CD8⁺ subsets isolation, and CFSE staining analysis. b – determination of the dividing T lymphocytes viability: CD3⁺ T cells isolation with subsequent dividing into CD4⁺ and CD8⁺ T lymphocytes subsets, gating of unstimulated sample, following the simultaneous analysis of vital Zombie UV and CFSE staining of stimulated sample

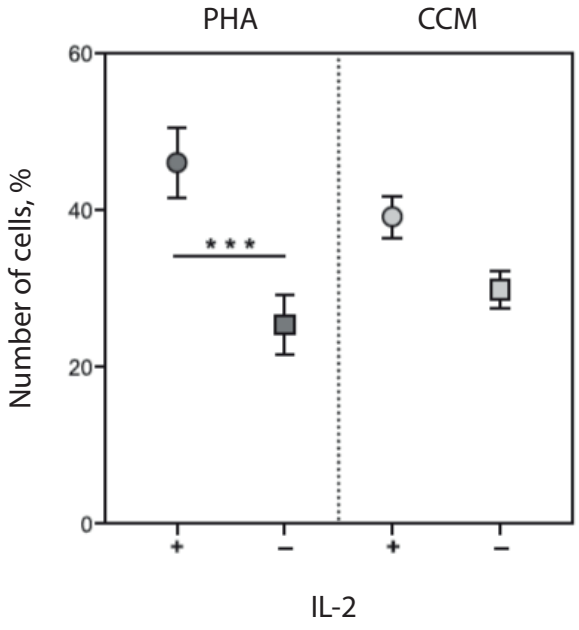

FIG. 3.
*The impact of adding IL-2 to the culture medium on the mononuclear leukocytes count following 7 days of incubation. The means and standard errors of the means are presented (n = 18 in each group). One-way analysis of variance was used to compare groups of quantitative data; multiple comparisons between groups were performed using Tukey's test; *** – p < 0.001*

(fig. 5a). It is important to note that the "rest" duration significantly affected the viability of stimulated cells: the differences between samples "H" and "O" were statistically significant ($p < 0.05$). At the same time, in samples "O" the proportion of surviving cells was reduced by more than 2 times compared to that in samples "H". In addition, it turned out that stimulated cells were more sensitive to the negative effects of "rest" than dormant elements. In cultures without the PHA addition, differences in cell numbers were revealed only between samples "I" and "O" ($p < 0.05$).

Analysis of the proliferative capacity of CD4⁺ and CD8⁺ T lymphocytes confirmed that "rest" is a negative factor. It was shown that the number of daughter generations formed by stimulated CD4⁺ and CD8⁺ T lymphocytes decreases when the "rest" stage of any time mode is included in the study protocol (fig. 5b).

It is noteworthy that the relative number of $CFSE^{low}$ cells that entered division among stimulated CD4⁺ and CD8⁺ T lymphocytes did not depend on the type of "rest" ($p > 0.05$; fig. 5c).

The introduction of an overnight "rest" into the thawing protocol significantly increased the percentage of dying cells among the CD4⁺ T lymphocytes that had entered division after being stimulated *in vitro*

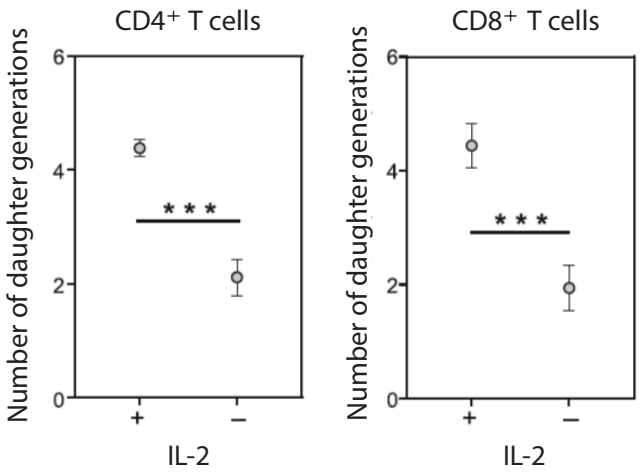

FIG. 4.
*The impact of adding IL-2 to the culture medium on the proliferation of CD4⁺ and CD8⁺ T lymphocytes during 7 days of in vitro stimulation. The means and standard errors of the means are presented (n = 18 in each group). Paired t-test was used to compare two groups of quantitative data; *** – p < 0.001*

for 7 days (fig. 5d). This phenomenon was not observed in the analysis of CD8⁺ T cells.

Thus, the inclusion of the "rest" stage in the mononuclear leukocyte thawing protocol followed by culturing stimulated cells for 7 days has a negative effect on the viability and proliferative capacity of CD4⁺ and CD8⁺ T lymphocytes. It is recommended to start working with cell cultures immediately after thawing cryopreserved mononuclear leukocytes.

DISCUSSION

This study raises a number of fundamental questions regarding the specific features of studying the functional activity of T lymphocytes using cryopreserved cells.

During the study, we determined that after 7 days of culturing cells thawed after long-term (40 ± 1.4 months) cryopreservation, on average only a third of their initially introduced number remains in the sample. Previously, when culturing mononuclear leukocytes that were not subjected to freezing, we did not note such a significant decrease in their number. An analysis of literature sources confirmed that a high tendency to die is a characteristic feature of cells that have undergone cryopreservation and thawing procedures [15].

It is known that freezing and thawing trigger processes leading to cell death – necrosis and apoptosis. Necrosis is a consequence of membrane damage by intracellular ice crystals and osmotic stress. Apoptosis can be caused by various factors, including physical stress caused by changes in cell morphology; activation of death receptors; accumulation of free radicals in the cytosol; activation of the caspase cascade [16, 17]. When using the optimal cryopreservation protocol, the main cause of death of thawed cells in culture is apoptosis [15]. It has been shown that a significant number of T lymphocytes constantly trigger the death program, which leads to a statistically significant reduction in the proportion of viable cells within 24 hours after the start of incubation. Based on the fact that stimulation of thawed cells with cytokines can significantly increase the viability of cultured cells [15], we investigated the effect of adding IL-2 to the culture medium on the functionality of T lymphocytes thawed after long-term cryopreservation.

IL-2, like other cytokines with a common gamma chain, is considered to be a survival factor for T lymphocytes *in vivo* and *in vitro* [18, 19]. It is known that IL-2 activates the tyrosine kinases Janus kinase (Jak) 1 and Jak3, which are adjacent to the cytoplasmic fragments of the cytokine receptor chains [20]. These enzymes phosphorylate tyrosine residues localized in the receptor chains, which creates sites for binding and phosphorylation of the adapter protein Shc and the transcription factor STAT5 (signal transducer and activator of transcription 5) [21]. Phosphorylated Shc activates several signaling pathways, at least one of which, PI3K/Akt (phosphatidylinositol 3-kinase/protein kinase B pathway), plays an important role in T cell proliferation [22]. In turn, phosphorylation of STAT5 molecules, their dimerization and translocation into the nucleus stimulate the expression of genes involved in division and protecting lymphocytes from apoptosis [23].

Our studies confirmed the positive effect of IL-2 added to the culture medium on the viability and proliferative activity of T cells thawed after cryopreservation. In the presence of this cytokine, we observed a less pronounced reduction in the number of leukocytes in the culture and an increase in the number of daughter generations during stimulation of T lymphocytes. However, the cultured cells still died en masse: their number significantly decreased after 7 days of incubation.

The most pronounced effect of IL-2 was on stimulated CD4⁺ and CD8⁺ T lymphocytes. Apparently, the higher sensitivity of these cells is due to the peculiarity of receptor expression that binds the cytokine. The high-affinity receptor for IL-2 is a complex of three chains: CD25, CD122 and CD132 [24]. Only two of them, CD122 and CD132, are expressed in limited quantities on resting T cells. By binding IL-2, they can form an intermediate-affinity complex capable of transmitting a signal into the cell. Stimulated T lymphocytes, in turn, induce the expression of CD25, which allows activated T cells to form a trimeric high-affinity receptor, making stimulated lymphocytes more sensitive to the cytokine action.

In the present study, we also assessed how the introduction of a "resting" step into the cell thawing protocol affects the functionality of cryopreserved T lymphocytes. Several studies [7–10], although not all [25], have previously demonstrated positive effects of "resting": a decrease in non-specific cytokine

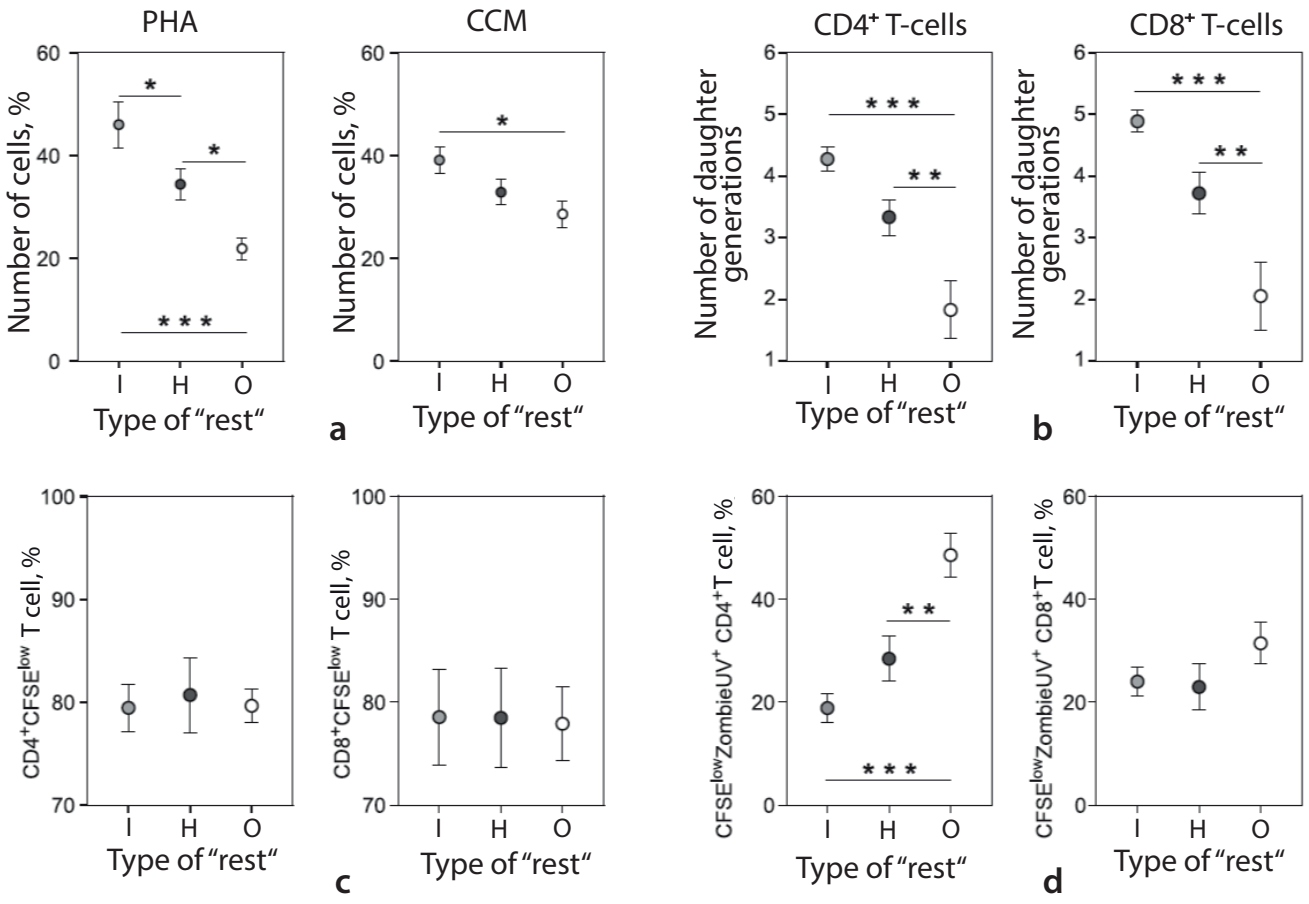

FIG. 5.

*Effect of the type of "rest" on the thawed cells parameters in a 7-day culture (the means and standard errors of the means are presented). a – number of mononuclear leucocytes in stimulated and control samples; b – number of daughter T cell generations in stimulated samples; c – number of proliferating T lymphocytes; d – number of T cells dying while dividing. Types of "rest": I – immediately after thawing (without "rest"; n = 18); H – hourly "rest" (n = 18); O – overnight "rest" (n = 18). One-way analysis of variance was used to compare groups of quantitative data; multiple comparisons between groups were performed using Tukey's test; * – p < 0.05; ** – p < 0,01; *** – p < 0.001*

production by T cells; an increase in the number of active T lymphocytes and the proportion of polyfunctional T lymphocytes. All of these phenomena were detected using the ELISPOT method. The results presented by the authors are apparently associated with the death of apoptotic T cells during the "resting" process, which contributes to an increase in the proportion of truly viable lymphocytes in the analyzed sample [9]. This is relevant given that the presence of apoptotic cells can reduce the functionality of live $CD4^+$ and $CD8^+$ T lymphocytes [26] and interfere with antigen processing [27].

As noted above, during cultivation, T cells thawed after cryopreservation constantly initiate programmed cell death processes [15], which was manifested in a gradual increase in the number of dead and dying T lymphocytes in the 7-day culture of mononuclear leukocytes that we set up. It can be assumed that the accumulation of apoptotic cells during longitudinal cultivation has a negative effect on the functional capacity of the remaining T lymphocytes, which was not noted in short-term studies using the ELISPOT method. Indeed, in our study, additional "rest" of cells (both hourly and overnight) led to a significant decrease in the number of leukocytes and an increase in the proportion of apoptotic T lymphocytes after 7 days of incubation. Moreover, we noted a decrease in the number of daughter generations among stimulated T lymphocytes that had undergone "rest" without changing the proportion of $CFSE^{low}$ cells. It is likely that in such cultures, compared to samples where the 'rest" stage was excluded from the work protocol, a greater number of cells enter into division, but they perform fewer mitoses.

Some limitations of the present study should be noted. Firstly, we did not reproduce the results of other research groups and therefore can only assume that "rest" has a positive effect in subsequent short-term studies (for example, using the ELISPOT method), but a negative

effect in further longer cell culturing (in our work, 7 days). Secondly, to modify the CCM, we used a single cytokine, IL-2, the effects of which can include not only stimulation of T lymphocyte proliferation, but also induction of regulatory T cells and initiation of activation-induced apoptosis of effector elements [28]. In further studies, we plan to introduce other cytokines of the IL-2 family into the culture medium, including IL-7 and IL-15, which are known to effectively support the viability and proliferation of T lymphocytes [29].

Despite the limitations, it should be emphasized that in the present study we evaluated for the first time the effect of "rest" on the results of proliferative capacity and viability assessment during the cultivation of T lymphocytes that had undergone long-term (40 ± 1.4 months) cryopreservation.

CONCLUSION

The obtained data encourage the development of new protocols for working with mononuclear leukocytes thawed after cryopreservation. It is obvious that the introduction of exogenous cytokines can increase the viability and functional activity of thawed T cells, bringing their state closer to that of freshly isolated lymphocytes. Optimal combination selection of cell culture medium components will allow obtaining data that more objectively reflect the processes occurring *in vivo*. It is also noteworthy that the "rest" of mononuclear leukocytes after thawing have a negative effect on the viability and proliferative activity of stimulated T lymphocytes during a week of incubation. This fact emphasizes the need to optimize research protocols.

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Conflicts of interest

No potential conflict of interest relevant to this article reported.

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Compliance with ethical standards

The study plan was approved by the Ethics Committee (registration no. IRB00008964; protocol no. 31 dated March 03, 2017). Each blood donor provided written informed consent. All procedures performed in the studies involving humans complied with the ethical standards of the national research ethics committee and the World Medical Association Declaration of Helsinki (1964) and its later amendments or comparable ethical standards.

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