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Improvement of bone marrow mononuclear cells cryopreservation methods to increase the efficiency of dendritic cell production

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ABSTRACT

Cryopreservation is now considered an integral part of the biotechnological process, exploiting different types of cells and tissues in clinical practice. Among them, dendritic cells (DCs) deserve special attention, notably the immature tolerogenic cells (tolDCs), which provide natural tolerance in humans and animals. High cryolability of tolDCs has necessitated the search for the methods that would provide cryopreservation of their precursors; those more resistant to negative effects of cryopreservation factors, in particular, bone marrow or peripheral blood mononuclear cells (MNCs). Based on this, the aim of our research was to optimize the cryopreservation conditions for mice bone marrow MNCs with further assessment of their ability to form tolDCs *ex vivo*. A cryopreservation mode for bone marrow MNCs has been developed which provides structural and functional completeness of tolDCs obtained from them *ex vivo*. The ability of DCs derived from cryopreserved MNCs by the developed mode to induce T-regulatory ($FOXP3^+$) cells *in vitro* when co-cultured with $CD4^+$-lymphocytes was shown. Tolerogenic properties of the DCs derived from cryopreserved MNCs are implemented by increasing the content of hsp70 heat shock proteins and the expression rate of glucocorticoid-induced leucine zipper (GILZ). DCs with increased tolerogenic activities, obtained by the developed cryopreservation regimen, can be used in treatment of autoimmune diseases.

In this research we not only evaluated the qualitative characteristics and tolerogenic activity of DCs produced *in vitro* from cryopreserved MNCs, but also outlined the prospects of accumulating their reserves in low-temperature banks for clinical use.

1. Introduction

In recent decades, the problem of the treatment of autoimmune diseases (AIDs) is one of the most vital. The pathogenesis of AIDs is based on deregulation of the immune system in the form of disordered tolerance to its own antigens and, as a result, activation and expansion of antigen-specific clones of T- and B-lymphocytes, production of circulating autoantibodies, cytokines and other mediators of inflammation [24]. The complexity, heterogeneity and multi-vector nature of autoimmune response minimizes the effectiveness of modern methods of AIDs treatment, which are mainly concentrated on systemic immune suppressive action, leading to severe side effects manifesting in a decreased resistance to infections, suppression of antitumor immunity, etc. [27]. The combination of all these challenging issues determines the need of searching the alternative ways of AIDs treatment. One of the promising therapeutic approaches is the use of *ex vivo* autologous tolerogenic DCs (tolDCs), capable of regulating the immune response, maintaining a balance between tolerance and immunity [15]. The mechanisms by which they implement the central and peripheral tolerance include, at a minimum, clonal deletion, anergy of T-effector lymphocytes and the induction of regulatory T-cells (Tregs) [18].

There are close relationships between tolDCs and Tregs both at the level of cell-to-cell cooperation and autocrine cytokine regulation [35]. It has been reported that the tolerogenic activity of DCs directly depends on the presence in the environment of IL-10, TGF-β, glucocorticoids, vitamin D3, retinoic acid, inhibiting their maturation [17]. However, the molecular mechanisms underlying this phenomenon remain unexplored. When using glucocorticoids to produce tolDCs, the ability of these cells to induce increased expression of an anti-inflammatory protein known as glucocorticoid-induced leucine zipper (GILZ) has been established [32].

GILZ transcription factor is considered a key link in the anti-

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inflammatory and immune suppressive effects of glucocorticoids. Increased expression of GILZ in DCs leads to NF-κB suppression and, consequently, an enhanced production of IL-10 when secretion of pro-inflammatory cytokines IL-12 and IL-23 is limited. In general, GILZ activation enhances the tolerogenic activity of DCs and the ability to stimulate Tregs formation [4]. Moreover, tolDCs, owing to IL-10, not only induce the formation of Tregs, but also support their production of the same cytokine, which confirms their cooperative interaction.

These properties of tolDCs are the basis for the development of a new therapeutic strategy to treat AIDs. The Tregs mentioned above, also designed to compensate the dysfunction of the T-regulatory link of the body in autoimmune pathology, can moreover be the candidates for the immune tolerance restoration. A number of studies have been devoted to the problem of cryopreservation of DCs, but the results obtained in them are very contradictory [14,20,29]. This may be stipulated by the fact that both MNCs of peripheral blood or bone marrow, isolated in the density gradient of certain solutions, and the DCs of different extents of maturity, obtained *ex vivo* from MNCs or monocytes, induced by the factors, can undergo cryopreservation.

Thus, in the report [29] the DCs derived from the MNCs of human or rats' spleen were frozen under the protection of 1 M or 2 M Me_2SO at different rates: 0.3; 1.5; 10; 20; 70; 150 deg/min to −60 °C followed by an immersion into liquid nitrogen. The authors found that the maximum preservation of DCs was observed at slow freezing rates, i.e. 0.3; 1.5 deg/min and it made below 57% for rat's DCs and 72% for human ones. The low level of preservation (not more than 40%) of DCs of C57Bl/6 mice after freezing at a rate of 1 deg/min to −70 °C, followed by immersion into liquid nitrogen, is evidenced by the results of Mendoza L. et al. [20].

Hayden H. et al. [14] demonstrated the freezing results for human peripheral blood monocytes and *in vitro* derived immature and semi-mature DCs under the protection of 10% Me_2SO at a rate of 1 deg/min to −80 °C. The most cryoresistant were monocytes, the viability of which was 90%, immature DCs showed 40% viability, semi-mature DCs did 20%. Each type of the above cells differs from the other both in structure and function that is a pre-requisite for different cryostability of cells.

In most studies, MNCs or monocytes were cryopreserved with a slow freezing program, specifically at the rate of 1 deg/min to −80 °C, followed by immersion into liquid nitrogen [3,14,19,23,26]. In these studies as components of the cryopreservation medium there were used 90% autologous or fetal bovine serum (FBS) and 10% Me_2SO or 40–70% RPMI-1640, 20–40% FBS and 10–20% Me_2SO. In particular, Hayden H. et al. [14] indicated that freezing of human peripheral blood monocytes in RPMI medium with 20% serum and 10% Me_2SO is more effective than with 90% serum. As evidenced by the results of a number of researches [14,15] the choice of warming temperature: room, for 10 min or 37 °C for 1 min, did not significantly affect a cell survival.

This protocol was developed to obtain functionally normal mature immune DCs for the treatment of some oncology pathologies [23,26,33]. However, in this case, there are many conflicting reports of a decrease in the rate of differentiation of cryopreserved MNCs in mature DCs upon stimulation of lipopolysaccharide (LPS) [15,20]. It was shown that in DCs obtained *ex vivo* from cryopreserved monocytes, the expression of CD83 and CD86 molecules was reduced, and the addition of LPS to the culture medium did not increase their production of IL-12 and TNFα pro-inflammatory cytokines. This is consistent with Messmer D. et al. [21] that the DCs derived from cryopreserved MNCs reduce the ability to produce a critical mediator of the inflammatory cascade, i.e. IL-12. Thus, the issue of effective cryopreservation of MNCs to subsequently obtain *ex vivo* tolDCs from them remains unresolved.

Assuming the task posed by these investigations, i.e. restoring the natural tolerance of the organism with AIDs, one cannot but dwell on the problem of Tregs cryopreservation. It should be noted that works on cryopreservation of *ex vivo* obtained Tregs are few, and the existing strategies for their cryopreservation have a number of limitations that need to be addressed before using in clinical practice. There are two approaches for Tregs cryopreservation [7]. One of them is based on the *ex vivo* production of Tregs from cryopreserved $CD4^+$ cells isolated by human peripheral blood leukapheresis. Another involves the freezing of Tregs themselves, which have the $CD4^+CD25^{hi}CD127^{lo/-}$ phenotype [6]. In both cases, there was a significant decrease in the viability of Tregs with a high percentage of necrotic and apoptotic cells both immediately after warming and after generation from cryopreserved $CD4^+$ cells. In addition, after cryopreservation, both in $CD4^+$ cells and among Tregs, a significant decrease in the percentage of $CD4^+CD25^{hi}CD127^-$ and $CD4^+FoxP3^+$ cells was found. The findings indicate the instability of the phenotypic characteristics of Tregs after cryopreservation that can significantly limit their functional activity [6]. In addition, the need for a repeated cultivation after cryopreservation of Tregs imposes certain difficulties for their use in clinical practice [7].

Based on the foregoing, it seems that DCs are a more preferred material than Tregs, both for cryopreservation and further use in adoptive therapy of AIDs. Based on this, the aim of the present research was to optimize the conditions for cryopreservation of bone marrow MNCs with further assessment of their ability to *ex vivo* form tolerogenic DCs.

2. Materials and methods

2.1. Animals

The experiments were carried out in 20-week-old CBA/H female mice weighing 24–26 g. The mice were kindly provided by Biomodelservice Company (Kyiv) and then raised under the standard conditions in the vivarium of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The study was conducted in accordance with "General Principles of Animal Experimentation" approved by the 5th National Congress on Bioethics (Kyiv, 2013) and in accordance with the regulations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986). The animals were sacrificed under ether anesthesia by cervical dislocation.

2.2. Isolation of bone marrow cells

Bone marrow (BM) was washed from murine femurs with RPMI-1640 medium (Biowest, France) containing 3% fetal bovine serum (FBS) (Biowest, France) and 2% sodium citrate (Sigma-Aldrich, USA) (hereinafter referred to as working medium) and re-suspended by passing through a needle with a decreasing diameter (0.7 mm, 0.6 mm, 0.55 mm). Afterwards, the BM cell suspension was passed through a nylon filter with 100 μm pores (Falkon, USA), centrifuged at 300 g for 10 min and re-suspended in working medium.

2.3. Isolation of bone marrow mononuclear cells

Mononuclear cells (MNCs) were isolated by Trazograph (Unique Pharmaceutical Laboratories, India) density gradient (1.077 g/mL) centrifugation of BM suspension [9].

2.4. Cryopreservation of bone marrow mononuclear cells

RPMI-1640 medium with 20% FBS and 20% Me_2SO (Arterium, Ukraine) was used as MNCs cryopreservation solution. Cryopreservation solution was added dropwise to MNCs suspension (obtained in working medium) in a ratio of 1:1 at room temperature (the final concentration of cryoprotectant was 10%) and the resulting mixture was of 6×10^6 cells/mL concentration in a 1.8 mL volume (totally 10.8×10^6 cells/plastic tubes (Nunc, Germany)). Cells were exposed to solution for 10 min at the same temperature. Mononuclear cells were frozen with a programmable freezer by two modes:

Mode 1 (M_1): the freezing rate was 1 deg/min to −80 °C followed by

immersion into liquid nitrogen (−196 °C);

Mode 2 (M_2): the same freezing rate to −40 °C followed by immersion into liquid nitrogen.

The choice of cryopreservation modes has been justified by the fact that M_1 is most often used for cryopreservation of MNCs or human peripheral blood monocytes [3], while M_2 allows passing the eutectic crystallization point of 10% Me_2SO solution (−66.7 °C) with a higher cooling rate (from −40 °C to −196 °C), in contrast to M_1, that reduces cryodestruction of cells during freeze-warming [12,13].

The samples were warmed in a water bath at 40–41 °C until the solid phase disappears. Warmed samples (1.8 mL) were washed once from Me_2SO by slow adding of two-fold volume (3.2 mL) of working medium and then centrifuged for 10 min (300 g). The precipitates in each group were diluted with a fresh portion of the working medium (1.8 mL) to assess the structural and functional completeness of MNCs.

Control samples (freshly isolated MNCs) were subjected to the same manipulations (exposure at room temperature for 10 min, centrifugation, adding the working medium to the precipitate) as the cryopreserved ones.

2.5. Structural and functional characteristics of cryopreserved MNCs

2.5.1. The number and viability of MNCs

Immediately after freeze-warming and washing from cryoprotectant the number and viability of MNCs were determined. MNCs cells washed from cryoprotectant in PBS and total number of cells were counted in Goryaev's chamber.

The MNCs viability was assessed in specimens with 1×10^6 cells in 100 μL PBS and then they were stained with propidium iodide (PI). After 15 min of incubation, 400 μL of PBS was added to the sample. The samples were immediately transferred to the flow cytometer (FACS Calibur, Becton Dickinson, USA) and analyzed. Unstained cells were used as control for viability assessing. The number of viable MNCs was determined by the formula:

$$\text{Viability},\% = 100\% - \text{PI}^+\text{-cells},\%$$

2.5.2. MTT assay

The metabolic activity of cryopreserved MNCs was assessed immediately after freeze-warming with cell growth determination kit MTT based (Stock No. CGD-1, Sigma, USA) according to the manufacturer's protocol. In brief, 1×10^6 MNCs in 200 μL cells per test tube were seeded with four parallels for each sample and MTT-Solution was aseptically added in an amount equal to 10% of the culture volume (20 μL). Test tubes were cultivated for 3 h in a humidified CO_2 incubator at 37 °C and 5% CO_2. After the incubation period, cultures were removed from incubator and the resulting MTT formazan crystals were dissolved by adding MTT-Solvent in an amount equal to the culture volume and incubated further for 1 h at room temperature in the absence of light. Finally, the formazan concentration was spectrophotometrically measured with a BioRad-680 microplate reader (PerkinElmer Lambda 465 PDA-UV-VIS, USA) at the 570 nm wavelength. Culture cell background absorbance was measured at 690 nm.

2.5.3. The detection of total protein in cells

The total protein in the MNCs lysate cells was determined using the Total Protein Kit (Rendox, UK). Optical density was measured with a CHEM 7 biochemical analyzer (ERBA Diagnostics Mannheim, Czech Republic) at 540 nm. The protein amount was calculated as per the manufacturer's instructions.

2.5.4. Flow cytometry analysis of MNCs

$CD14^+$-monocytes of cryopreserved MNCs just after freeze-warming and washing from cryoprotectant were counted with the flow cytometer using an anti-mouse monoclonal antibody (mAbs): CD_{14} FITC (BD Biosciences, USA), the content of $hsp70^+$ cells was measured using anti-hsp70 mAbs (FITC) (Abcam, UK) in compliance with the manufacturer's protocol. Fluorochrome-positive (FITC) cells were determined using isotypic controls (BD Bioscience, USA and Abcam, UK). The isotype-control was level of non-specific background signal caused by primary antibodies. These values were calculated for all antibodies at the initial set up.

Staining protocol was as follows: cell suspensions with a concentration of 1×10^6 in 100 ML of PBS were incubated for 30 min at room temperature (25 °C) with 2 μL FITC-labeled mAbs. After exposure to mAbs, the cells were washed by adding 100 mL of PBS, followed by centrifugation (300 g) for 10 min, then was added 400 μL of PBS to the sediment. The content of labeled cells was assessed with a FACS Calibur flow cytometer using the CellQuest Pro Software (BD Biosciences). In each sample, 10,000 events were analyzed. The data obtained were assessed using the WinMDI 2.8 software. (Joseph Trotter, Scripps Institute, La Jolla, USA), determining the percentage of cells positive for each mAbs. In native controls these parameters were measured after the same manipulations as with cryopreserved specimens.

2.6. Structural and functional characteristics of tolerogenic DCs derived in vitro from cryopreserved MNCs

2.6.1. Generation of DCs from cryopreserved MNCs

MNCs at a concentration of $3–5 \times 10^6$ cells/mL were cultured in 3 cm plastic Petri dishes in 3 mL of RPMI-1640 medium with 10% FCS and 1% antibiotic solution (100 U/mL of penicillin, 0.1 mg/mL of streptomycin). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. After 2 h culturing MNCs the medium with unattached cells was removed. Then on day 0, recombinant murine GM-CSF (Sigma-Aldrich, UK) (20 ng/mL), IL-4 (Sigma-Aldrich, UK) (5 ng/mL) and dexamethasone - Dex (Sigma-Aldrich, UK) (0.4 μg/mL) were added to the culture medium to obtain tolDCs [9,16]. After 3 days, another 3 mL of fresh culture medium with the same components were added to each Petri dish. On day 7, non-adherent, being the candidate for immature tolDCs [5,9,35] were harvested from dishes, centrifuged at 300 g for 10 min, re-suspended in working medium and leveled to 1×10^7 cells/mL. for further flow cytometry analysis and assessment of the DCs functional potential.

2.6.2. Flow cytometry analysis of DCs

Phenotype of DCs obtained after culturing of cryopreserved MNCs for 7 days was determined with flow cytometer using mAbs: CD11b (FITC), CD14 (FITC), CD83 (PE), CD80 (FITC), CD86 (FITC) (BD Bioscience, USA) and hsp70 (FITC) (Abcam, UK) accordingly to the manufacturer's protocols by flow cytometry. The cells were supplemented with 2 μL of FITC-labeled mAbs or 5 μL of PE-labeled mAbs. The percentage of cells positive for each mAbs and the mean fluorescence intensity (MFI) was examined according to the Gmean index. Indies of DCs derived *in vitro* from native MNCs were measured as controls.

2.6.3. Content of GILZ gene transcripts

The *GILZ* gene expression in DCs obtained after culturing of native and cryopreserved MNCs with DCs inducers for 7 days was assessed by real-time PCR with reverse transcription. Total RNA was extracted from 5×10^6 cells using the Thermo Scientific GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. To isolate genome DNA all the samples were treated with DNAase (Sintol, Russia): 5 μl of DNAase was added in reactive mixture and incubated at 37 °C for 30 min, then at 75 °C for 10 min inactivate the enzyme.

Reverse transcription was performed using the Thermo Scientific First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per the manufacturer's protocol. The obtained cDNA was immediately used for PCR or, if necessary, stored at – 20 °C.

Real-time PCR was performed with ANK-16 analyzer (Sintol, Russia) using the Maxima SYBR Green/ROX qPCR Master Mix (2X) kit

(ThermoScientific, USA). The final reaction mixture for amplification contained SYBR Green dye, Maxima Hot Start Taq DNA Polymerase, 0.2 μl of forward and reverse specific primers, and 1 μl of template (cDNA). The reaction mixture was brought to a total volume of 25 μl by adding deionized H_2O. The specific primer pairs for analysis of the studied and reference genes were selected using the Primer-Blast tool (www.ncbi.nlm.nih.Gov/tools/primer-blast) and manufactured by Metabion (Germany) (see Table 1). After the initial 10-min denaturation at 95 °C, the amplification consisted of 40 cycles and was performed under the following conditions: denaturation at 95 °C, 15 s; annealing at 59–61 °C, 30–60 s; elongation at 72 °C, 30 s. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a reference gene to detect relative changes in the expression of the genes under investigation.

Replicates were made for each analyzed sample; negative controls were also set. Fluorescence was measured automatically during PCR. Expression was analyzed using the method of delta-delta Cp (ΔΔcp) by the following formula:

$$R = \frac{(E\ \ GILZ)^{\ \Delta cp\ \ GILZ\ \ (natDC\ -\ \ experiment)}}{(E\ \ GAPDH)^{\ \Delta cp\ \ GILZ\ \ (natDC\ -\ \ experiment)}}$$

where R – relative expression of the target gene (*GILZ*); E_{GILZ} and E_{GAPDH} – PCR efficiency of cDNA of the target gene (*GILZ*) and the *GAPDH* gene, respectively; Δcp *GILZ* and Δcp *GAPDH* – deviations of cp (natDC - experiment) of cDNA of the *GILZ* and *GAPDH* genes, respectively.

2.7. In vitro evaluation of DCs tolerogenic activity by their ability to induce Tregs from $CD4^+$ spleen cells

Isolation of $CD4^+$ cells from the spleen of CBA/H mice with adjuvant arthritis (AA, 14 day) was performed with a magnetic sorter (BDTM Imagnet) using anti-mouse CD4 Magnetic Particles - DM (BD) according to the manufacturing protocol. The purity of $CD4^+$ cells was 82 ± 12%. Adjuvant arthritis was induced in CBA/H mice by subplantar administration of complete Freund's adjuvant (Santa Cruz, USA) at a dose of 0.1 mL/mouse [1,10].

2.7.1. Induction of Tregs in the co-cultivation of DCs with $CD4^+$ cells

Freshly isolated $CD4^+$ spleen cells (2.5×10^6) of mice with AA were co-cultured with DCs (0.5×10^6) obtained from cryopreserved MNCs in RPMI-1640 medium with 10% FBS without cytokines for 4 days in a CO_2 incubator with 5% CO_2 at 37 °C [14]. The supernatant with non-adherent cells of the experimental and control groups was afterwards transferred to centrifuge tubes and precipitated by centrifugation at 300*g* for 10 min. The precipitated cells were re-suspended in PBS to 1×10^6 cells per 100 μl.

Table 1
Characteristics of the primers to the *GILZ* and *GAPDH* genes (*GAPDH* gene is a household gene).

Gene name and Genbank accession number	Forward (F) and reverse (R) primers	Length of amplification product
GILZ		
Mus musculus TSC22 domain family, member 3 (Tsc22d3), transcript variant 1, mRNA NM_001077364.1	F:GTCCCAAGGACATGCCCATCTG R: ACAGTCATTGTCAGGTGAAGCTG	276 bp
GAPDH		
Mus musculus glyceraldehyde-3-phosphate dehydrogenas, transcript variant X1, mRNA XM_017321385.1	F:GCCTTCCGTGTTCCTACCC R:CAGTGGGCCCTCAGATGC	1813 bp

Cells appurtenance to Treg were examined by the ability to express FOXP3 marker. Cells were permeabilized using the BD Cytofix/CytopermTM Fixation/Permeabilization Kit. The amount of Tregs ($FOXP3^+$) was determined by fluorescence using mAbs to FOXP3 (PE) (Becton Dickinson, USA) according to the manufacturer's protocol. The control was the content of $FOXP3^+$ in $CD4^+$ cells of the spleen, which were cultured without DCs.

2.8. Statistical data processing

Data were processed by a nonparametric statistical method in the Statistica 10.0 software (StatSoft, Inc., USA) and the results are presented as medians with lower and upper quartiles (Me [LQ; UQ]) according to the International System of Units recommended for use in clinical and laboratory practice. The Kruskal-Wallis test was used for multiple comparisons of independent samples (differences were considered significant at $p < 0.05$). The groups ($n = 5$ in each group) were pairwise compared using a nonparametric Mann-Whitney test with Bonferroni correction (differences were considered significant at $p < 0.01$).

3. Results and discussion

3.1. Number and viability of MNCs after cryopreservation

Evaluation just after warming and washing from cryoprotectant of the cryopreserved with different modes MNCs demonstrated that the number (Table 2) and viability of $cryoM_2MNCs$ (Fig. 1) were higher compared to $cryoM_1MNCs$. So, number and viability of MNCs after freezing by M_2 did not have strong differences if compared with natMNCs, meanwhile when M_1 was used these indices (number and viability) decreased versus natMNCs by 24% and 20%, correspondingly.

3.2. Metabolic activity of MNCs after cryopreservation

The MTT system is a simple, accurate, reproducible means of measuring the activity of living cells via mitochondrial dehydrogenase activity. The key component is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increased cell number results in a rise in the amount of MTT formazan formed and an enhanced absorbance. However, our results demonstrated no rise of metabolic activity of cells immediately after freeze-warming, but M_2 still had an advantage over M_1 in this index (Fig. 2). In $cryoM_1MNCs$ just after warming the metabolic activity was reduced by 30%, while in $cryoM_2MNCs$ it did not differ from natMNCs.

Table 2
The number of MNCs cryopreserved under different modes (M_1 ore M_2) immediatelly after freeze-warming and washing from a cryoprotectant, Me [LQ; UQ].

Groups	Count, $x10^7$
natMNCs (control)	0.96 [0.94; 1.10]
$cryoM_1MNCs$	0.73 [0.71; 0.75]*
$cryoM_2MNCs$	0.85 [0.84; 0.94]#

Note: * - significant differences compared to natMNCs; # - significant differences compared to $cryoM_1MNCs$ ($p < 0.01$, Bonferroni-corrected Mann-Whitney *U* test).

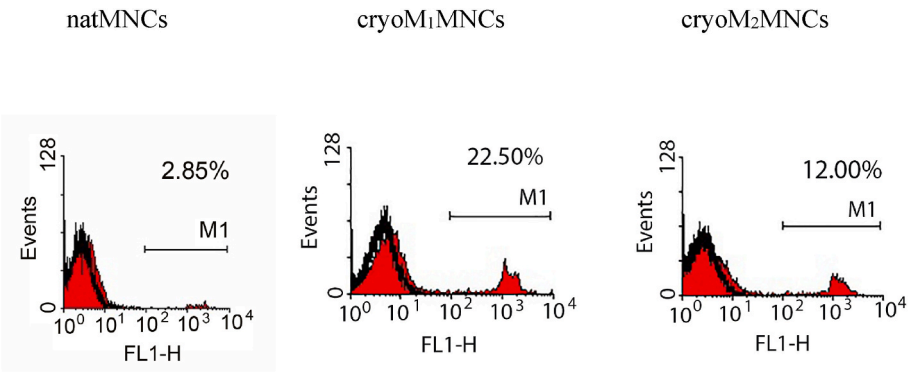

Fig. 1. Representive flow cytometry histogrames of PI $^{+}$ MNCs under cryopreservation by different modes (M_1 or M_2) immediatelly after freeze-warming and washing from a cryoprotectant. Notes: unstained cells were used as control for viability assessing. The number of viable MNCs was determined by the formula: Viability, % = 100% - PI^{+}cells, %. For natMNCs, the viability was 97.15%; for cryoM_1MNCs and cryoM_2MNCs - 77.5% and 88.00%, respectively.

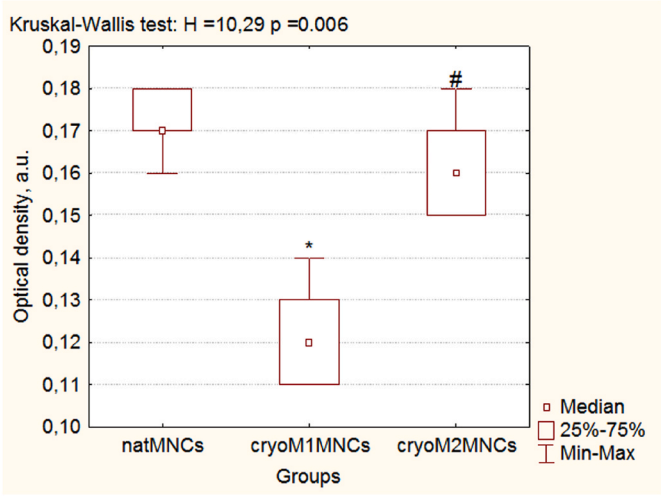

Fig. 2. Metabolic activity of MNCs cryopreserved under different modes immediatelly after freeze-warming. Note: * - significant differences compared to natMNCs; # - significant differences compared to cryoM_1MNCs ($p < 0.01$, Bonferroni-corrected Mann-Whitney *U* test).

3.3. The content of $CD14^{+}$cells in cryopreserved MNCs and DCs derived from them in vitro

Monocytes being a part of MNCs and expressing the phenotypic marker CD14, are known to be precursors of DCs. It was found that the pre-cryopreservation percentage of CD 14^{+} cells in the natMNCs was 12.30% (Fig. 3), whereas immediately after freeze-warming and washing from a cryoprotectant of cryoM_2MNCs, the percentage of $CD14^{+}$ cells was significantly higher (18.00%), both in comparison with the native control and with cryoM_1MNCs (15.80%). However, we can not ignore the increased percentage of $CD14^{+}$ cells in the cryoM_2MNCs occurred not only because of the death of other cells (the same was also found by M_1), but also due to the expression of this marker on CD14precursors of monocytes. Similar trends were noted for other markers after cryopreservation of heterogeneous cell suspensions [8,30].

To obtain immature tolDC, cryopreserved MNCs with GM-CSF and IL-4 were cultured for 7 days. The percentage of $CD14^{+}$ monocytes in the natDCs, cryoM_1DCs and cryoM_2DCs groups was significantly lower if compared with the natMNCs, cryoM_1MNCs and cryoM_2MNCs groups (2.92-, 1.90- and 1.88-fold, respectively). (Fig. 3). Our data are consistent with Silveira et al. [26] who also observed inhibition of the transition of $CD14^{+}$ monocytes into immature DCs after cryopreservation due to reduction in the expression of GM-CSF (CD116) and IL-4 (CD124) receptors.

3.4. Flow cytometry analysis of phenotype of DCs derived in vitro from cryopreserved MNCs

Certain phenotypic markers of cells may indicate the degree of their transition to immature DCs. Most often, immature DCs include cells with a low expression of MHC I and II class molecules, co-stimulatory molecules (CD40, CD80 and CD86) and with a minimal amount of CD83, a typical marker of mature DCs [9]. At the same time, the integrin CD11b is only present on immature tolDCs [15], as tolDCs need this molecule to inhibit Th17 differentiation and T cell activation [34].

Flow cytometry analysis showed that the percentage of $CD11b^{+}$ cells among natDCs on day 7 of cultivation derived *in vitro* from natMNCs was 85.4%; the percentage of $CD80^{+}$ and $CD86^{+}$ cells – 55.8% and 70.3%, respectively; the percentage of $CD14^{-}CD83^{+}$ cells – 8.7% (Table 3). It is known that the expression level of a membrane marker determines the functional potential of cells [10,35], as well as that the tolerogenic potential of immature DCs depends on the expression of the CD11b marker. Based on this, the results of evaluation of the percentage of $CD11b^{+}$ cells and of their mean fluorescence intensity (MFI, 215.3 arbitrary units) (Table 3) as well as a low count of $CD14^{-}CD83^{+}$ cells grown from natMNCs can be an evidence of the fact that immature, tolDCs were obtained in culture. The percentage of $CD80^{+}$ and $CD86^{+}$ cells and their MFI are consistent with other researchers' data [35], where similar protocols were used to obtain immature DCs. It is noteworthy that Yang J. et al. [35] reported that the amounts of $CD80^{+}$ and $CD86^{+}$ cells in a composition of mature and tolDCs populations barely differed, however, the MFI of these markers in tolDCs was lower by 5 and 27 times, respectively, compared with mature immune DCs.

After culturing the cryopreserved for 7 days MNCs with DCs inducers, the content of $CD11b^{+}$, $CD80^{+}$ and $CD86^{+}$ cells changed compared to natDCs (Table 3). Thus, the content of $CD11b^{+}$ in cryopreserved DCs was lower versus natDCs, but their MFI increased significantly. It is important that the content of $CD11b^{+}$ cryoM_2DCs in comparison with cryoM_1DCs was higher (67.10% and 53.30%, respectively). Importantly, regardless of the mode, in cryopreserved DCs the content of $CD80^{+}$ and $CD86^{+}$ cells was significantly reduced compared to natDCs, and in cryoM_2DCs that was to a greater extent. This indicates a limitation of cryopreserved DCs ability to cooperatively interact with T-effector cells, i.e. immune function. It is noteworthy that after cryopreservation, the ratio of formed cells expressing purely tolDCs marker ($CD11b^{+}$) and those belonging to mature DCs ($CD14^{-}CD83^{+}$, $CD80^{+}$, $CD86^{+}$) changed. This value was 0.63 for natDCs, 0.83 for cryoM_1DCs, and 1.29 arb. units for cryoM_2DCs, also demonstrating an increase in the cryoM_2DCs tolerogenic potential. Taken together, these data show the advantage of the M_2 cryopreservation over M_1 to obtain tolDCs.

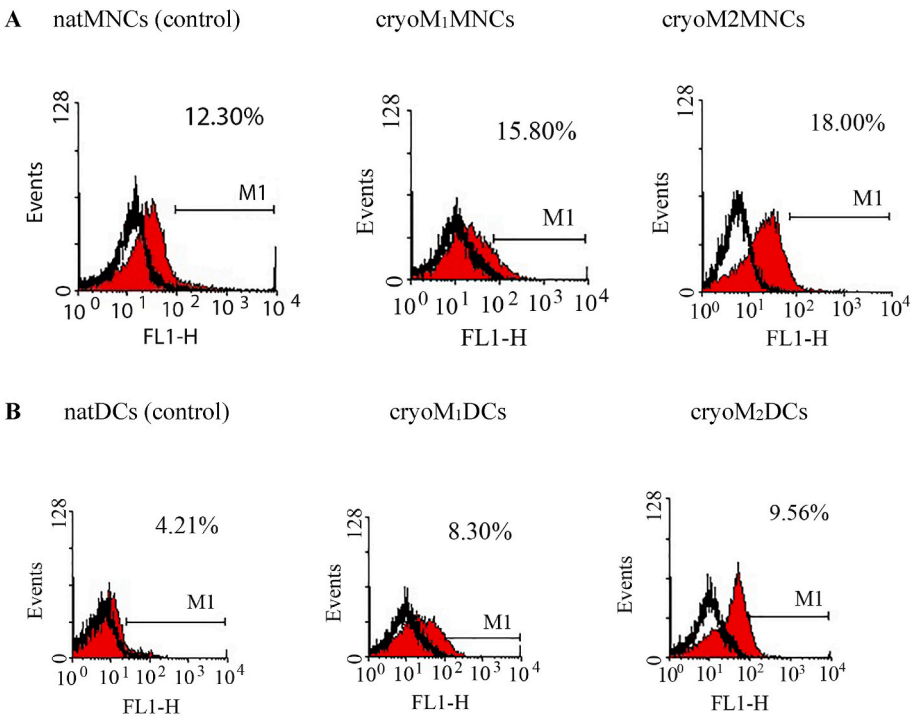

Fig. 3. Representive flow cytometry histogrames of the content of $CD14^+$cells in MNCs immediatelly after freeze-warming and washing from a cryoprotectant (A) and after 7-day cultivation with inducers of DCs (B).

Table 3
Features of expression of the phenotypic markers of DCs derived *in vitro* from natMNCs, cryoM_1MNCs and cryoM_2MNCs after 7 days of their cultivation with inducers of immature DCs, Me [LQ; UQ].

Expressing marker	Source of DCs					
	natDCs from natMNCs		cryoM_1DCs from cryoM_1MNCs		cryoM_2DCs from cryoM_2MNCs	
	Percentage of cells	MFI, arbitrary units	Percentage of cells	MFI, arbitrary units	Percentage of cells	MFI, arbitrary units
$CD11b^+$	85.4 [83.7; 86.4]	215.3 [197.6; 222.4]	53.3 [51.9; 55.3]*	871.4 [854.3; 899.2]*	67.1 [62.2; 68.8]*#	864.4 [854.4; 914.3]*
$CD80^+$	55.8 [54.6; 56.3]	281.3 [269.4; 287.1]	26.3 [26.1; 28.3]*	339.7 [327.1; 341.4]*	19.7 [19.4; 21.6]*#	346.2 [324.2; 369.4]*
$CD86^+$	70.3 [65.3; 71.3]	239.4 [231.4; 241.6]	29.7 [27.2; 31.4]*	283.6 [261.1; 287.3]*	23.5 [21.3; 23.8]*#	309.2 [285.4; 324.1]*
$CD14^-CD83^+$	8.7 [8.3; 8.9]	342.5 [335.1; 369.3]	8.2 [7.7; 8.3]	371.3 [354.3; 378.4]	6.9 [6.8; 7.2]*#	357.3 [352.1; 369.5]

Note: MFI – mean fluorescence intensity; * – significant differences compared to natDCs; # – significant differences compared to cryoM_1DCs ($p < 0.01$, Bonferroni-corrected Mann-Whitney *U* test).

We have previously emphasized that by varying cryopreservation conditions, it is possible to change both the structural characteristics and functional potential of certain cells and tissues, which may affect the effectiveness of their use for therapeutic purposes [8,11]. Our results would also indicate that the changes in the receptor assortment of DCs derived from MNCs also depend on the cryopreservation conditions.

3.5. *The content of hsp70$^+$ cells in cryopreserved MNCs and DCs derived from them in vitro*

Heat shock proteins, in particular the hsp70 family, is of special focus in functional changes to cells after cryopreservation. Proteins of this family act as activators of anti-inflammatory processes. It is assumed that the mechanism of immunosuppressive action of hsp70 is implemented through transforming the phenotype and function of myeloid suppressors, monocytes and DCs into tolerogenic ones, including their production of IL-10 [2]. This can be confirmed by the fact of cessation of DCs differentiation, manifested as reduced expression of membrane MHC II, as well as of co-stimulatory molecules CD80/86 and their acquisition of suppressor function after *in vitro* incubation with microbial hsp70 [22,28].

The data presented in Fig. 4 show the content of $hsp70^+$ cells in cryopreserved MNCs preserved under different modes. It was found that M_2 contributed to a more conspicuous increase in the content of $hsp70^+$ cells in cryopreserved MNCs immediately after warming and washing from cryoprotectant than M_1 (2.9- and 1.7-fold, respectively) when compared to natMNCs (Fig. 4A). This may be a trigger for a rise in the number of immature tolDCs. Indeed, after 7-day cultivation of natMNCs (Fig. 4B), the percentage of $hsp70^+$ cells was 2.77%, which did not significantly differ from that obtained from cryoM_1MNCs. However, after 7-day cultivation, there was nearly twice increased in the percentage of $hsp70^+$ cells formed from cryoM_2MNCs in comparison to natMNCs and cryoM_1MNCs. Hence, M_2 contributed to a rise in tolerogenic activity of MNC-derived DCs.

3.6. *The relative expression of the GILZ gene in DCs*

The formation of tolerogenic properties in DCs is associated with various factors. Immunosuppressive agents, such as glucocorticoids, are most commonly used to induce tolDCs. *In vivo* models of inflammatory diseases in animals and *in vitro* experiments have shown that the anti-inflammatory activity of glucocorticoids is realized through the

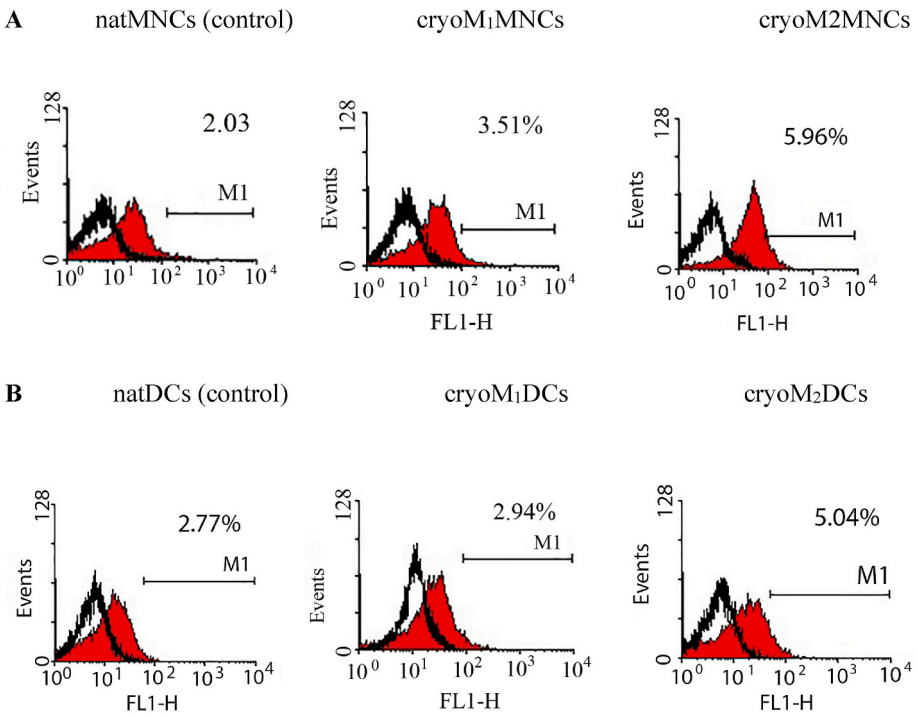

Fig. 4. Representive flow cytometry histogrames of the content of hsp70$^+$ cells in MNCs immediatelly after freeze-warming and washing from a cryoprotectant (A) and after 7-day cultivation with inducers of DCs (B).

expression of *GILZ* [25], which clearly identifies *GILZ* as a common intracellular marker for tolDCs. Increase in its expression inhibits the maturation and implementation of the presenting function of DCs [32]. The issue of the cryopreservation effect on the *GILZ* expression remains of great interest. Of course, in this case we are discussing cryopreservation as a powerful stress-inducing factor that allows for activation/inhibition of the expression of genes and postgenomic cascade pathways responsible for the *GILZ* production. In this regard, we evaluated the relative expression of the *GILZ* gene, which regulates the tolerogenic properties of DCs obtained in cryopreserved MNCs culture (Fig. 5).

The data indicate that the *GILZ* gene expression was activated in DCs obtained from cultures of both cryoM$_1$MNCs and cryoM$_2$MNCs. The maximum of mRNA transcripts of this gene was observed in cryoM$_2$DCs, exceeding the R in natDCs and cryoM$_1$DCs by more than 2 and 1.3 times, respectively. In view of this, there is reason to believe that cryopreservation specifically induces the immature phenotype of DCs to be formed, limiting chances for their further maturation. This may be due to shedding or to decreased expression of TLR4 and other receptor structures after warming. These data are confirmed by Silveira et al. [26], who showed that, after cryopreservation, LPS-stimulated monocytes produced mature DCs with a significantly lower intensity than native cells and retained the immature phenotype. Our results on activation of *GILZ* gene expression in DCs exhibit well that cryopreservation can act as a factor promoting the acquisition of the tolerogenic potential by DCs.

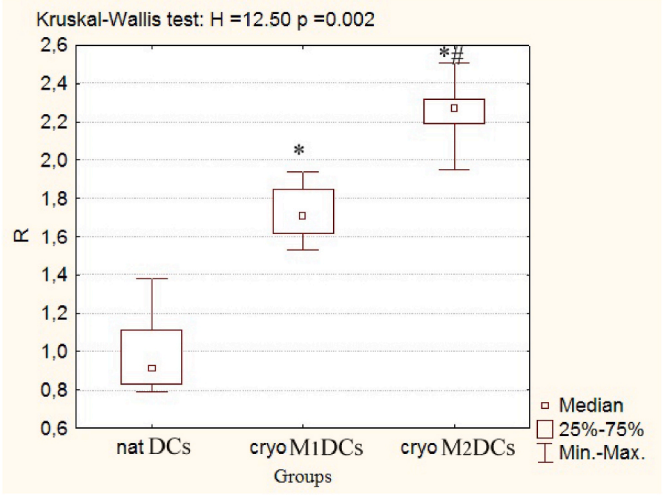

Fig. 5. The relative expression (R) of the *GILZ* gene in natDCs, cryoM$_1$DCs and cryoM$_2$DCs obtained of natMNCs, cryoM$_1$MNCs and cryoM$_2$MNCs after 7-day cultivation with inducers of DCs. Notes: the R in natDCs was taken for 1; * - significant differences compared to natDCs; # - significant differences compared to cryoM$_1$DCs ($p < 0.01$, Bonferroni-corrected Mann-Whitney *U* test).

3.7. *In vitro evaluation of DCs tolerogenic activity*

One of the most convincing criteria for the DCs tolerogenic activity is known to be the formation of Treg cells under their influence [5,31]. Our data (Fig. 6) show that among freshly isolated CD4$^+$ cells of the spleen of mice with AA cultured without DCs (control), the content of Tregs (FOXP3$^+$ cells) was 4.24%. This value increased in 1.4 times when co-culturing the CD4$^+$ spleen cells with natDCs (6.01%), while that with cryoM$_1$DCs it enhanced in 3.5 times (14.94%). Our results are consistent with data of Hayden H. and co-authors [14], who also noted the stimulation of CD4$^+$FOXP3$^+$ Tregs formation when co-culturing human peripheral blood CD3$^+$ cells with DCs grown from cryopreserved monocytes that coincided with the M$_1$ we used. Co-culturing CD4$^+$ spleen cells with cryoM$_2$DCs showed a five-fold rise of FOXP3$^+$ cells (22.18%) compared to the controls. This fact indicates an increased tolerogenic activity of cryoM$_2$DCs compared to cryoM$_1$DCs.

4. Conclusions

We have demonstrated that cryopreservation of MNCs with freezing rate of 1 deg/min to −40 °C followed by immersion into liquid nitrogen (−196 °C) under protection of 10% Me$_2$SO ensured their higher

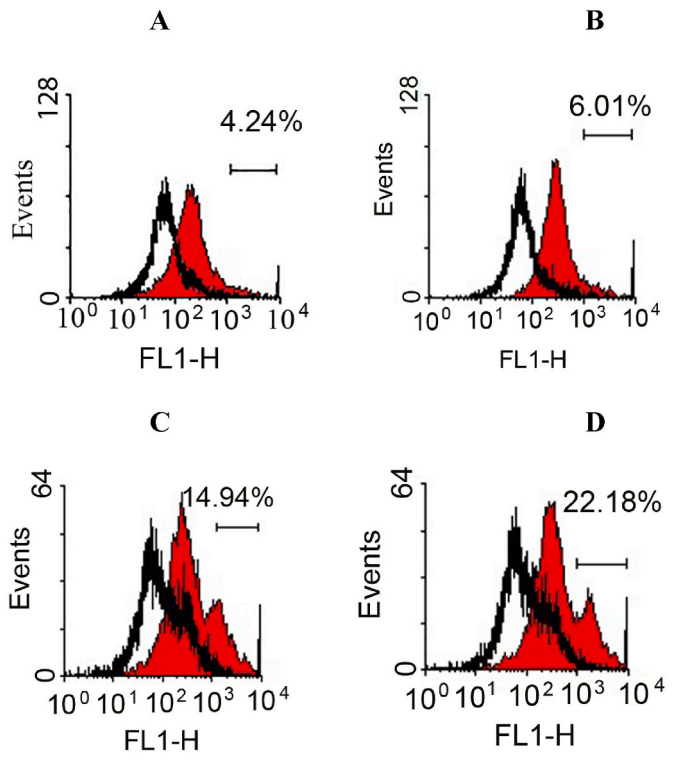

Fig. 6. Histograms presenting the content of FOXP3$^+$ cells, formed from CD4$^+$ spleen cells of animals with AA after their co-cultivation *in vitro* with natDCs (B), cryoM$_1$DCs (C), cryoM$_2$DCs (D). The control was the content of FOXP3$^+$ cells in CD4$^+$ spleen cells cultured without DCs (A).

viability, metabolic activity and total number as well as greater content of CD14$^+$ cells in comparison with M$_1$. These results confirm that cryopreservation can act not only as a technology for long-term storage of different types of cells, tissues, etc., but also as a method for managing their internal state. Analysis of both the *GILZ* gene expression and the hsp70 content in DCs together with the evaluation of phenotypic markers and tolerogenic activity can predict their therapeutic potential for treatment of autoimmune pathologies.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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