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JUSTIFICATION OF THERAPEUTIC EFFICACY OF DENDRITIC CELLS DERIVED FROM CRYOPRESERVED PRECURSORS IN AN ADJUVANT ARTHRITIS MODEL

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Tolerogenic dendritic cells are of particular interest in the search for new approaches to the correction of the T-regulatory immunity circuit in the treatment of autoimmune diseases. The aim of this study was to investigate the efficacy of dendritic cellsderived from cryopreserved bone marrow mononuclear cells in the formation of a tolerogenic platform in the recipient through the mechanism of stimulating T-regulatory cells in an experimental model of adjuvant arthritis. Dendritic cells were obtained *in vitro* from cryopreserved mononuclear cells. On the 14th day of pathology development, various types of tolerogenic dendritic cells were administered to the animals; the content of T-regulatory cells ($CD4^+CD25^+$ and $FOXP3^+$) and the level of *foxp3* gene expression in the recipient's spleen cells were evaluated. The developed method of cryopreservation of mononuclear cells, based on theoretical justification, provided high tolerogenic activity of dendritic cells derived from cryopreserved precursors. As a result, there was an increase in the content of T-regulatory cells and their functional potential through the activation of the *foxp3* gene. This led to the normalization of the main clinical diagnostic indicator – the arthritis index.

Keywords: cryopreservation of bone marrow mononuclear cells, tolerogenic dendritic cells, T-regulatory cells, *foxp3* gene, adjuvant arthritis.

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ОБГРУНТУВАННЯ ЛІКУВАЛЬНОЇ ЕФЕКТИВНОСТІ ДЕНДРИТНИХ КЛІТИН, ОТРИМАНИХ З КРІОКОНСЕРВОВАНИХ ПОПЕРЕДНИКІВ, В МОДЕЛІ АД'ЮВАНТНОГО АРТРИТУ

Толерогенні дендритні клітини становлять особливий інтерес у світлі пошуку нових підходів до корекції Т-регуляторної ланки імунітету при лікуванні аутоімунних захворювань. Метою роботи було дослідити ефективність дендритних клітин, отриманих з кріоконсервованих мононуклеарів кісткового мозку, в формуванні толерогенного плацдарму реципієнта через механізм стимулювання Т-регулятирних клітин в експериментальній моделі ад'ювантного артриту. Дендритні клітини отримували *in vitro* з кріоконсервованих мононуклеарів. На 14-у добу розвитку патології тваринам вводили різного виду толерогенні дендритні клітини і оцінювали вміст Т-регуляторних клітин ($CD4^+CD25^+$ та $FOXP3^+$) та рівень експресії гена *foxp3* в клітинах селезінки реципієнтів. Розроблений на підставі теоретичного обгрунтування спосіб кріоконсервування мононуклеарів на відміну від загальноприйнятого забезпечував високу толерогенну активність дендритних клітин, отриманих з кріоконсервованих попередників, результатом якої було підвищення вмісту Т-регулятиних клітин і їх функціонального потенціалу за рахунок активації гена *foxp3*. Це призводило до нормалізації основного клініко-діагностичного показника – індексу артриту.

Ключові слова: кріоконсервування мононуклеарів кісткового мозку, толерогенні дендритні клітини, Т-регуляторні клітини, ген *foxp3*, ад'ювантний артрит.

The study is a fragment of the research project "Study of the Activation Mechanisms of Tolerogenic Activity of Dendritic Cells under the Influence of Cryopreservation and Components of Umbilical Cord Blood", state registration No. 0117U000852.

Currently, autoimmune diseases (ADs) rank third among the most prevalent diseases worldwide. They are classified as multifactorial disorders, although the main cause is considered to be the breakdown of natural tolerance of the immune system to self-antigens [1]. AD triggers can also include changes in the antigenic spectrum of the body's own tissues and the formation of immunogenic epitopes (epitope spreading). This can be caused by persistent metabolic disturbances in tissues, prolonged infectious burden accompanied by chronic inflammation, which leads to the generation of reactive oxygen species (ROS). The chronic exposure of tissues to ROS contributes to the formation of self-immunogenic epitopes and can be a risk factor for the development of ADs [12].

The evident necessity for developing a treatment strategy for autoimmune diseases (ADs), particularly rheumatoid arthritis (RA), is well recognized [2]. It is known that T-regulatory cells (Tregs) play a significant role in suppressing the autoimmune response to self-antigens; their content and function are diminished in RA [4, 11]. The level of suppressive activity of Tregs is largely determined by the expression of the transcription factor forkhead box protein 3 (FOXP3) gene, which is essential for the normal functioning and development of Tregs [4, 10]. High expression of FOXP3 has been demonstrated in $CD25^+CD4^+$ Tregs. However, in RA patients, a decrease in the number of suppressor cells with active

foxp3 gene expression has been observed [10], possibly due to an imbalance in the production of pro- and anti-inflammatory cytokines [1, 7, 13]. Inducing antigen-specific tolerance, in which tolerogenic dendritic cells (tolDCs) play a crucial role, is a promising approach for correcting such disturbances [2, 5, 11]. The use of tolDCs in autoimmune diseases therapy requires the development of efficient cryopreservation and storage technologies to ensure their availability when needed. Since tolDCs are highly vulnerable to cryopreservation factors, it is necessary to search for more resilient precursor cells from which functional tolDCs can be derived *in vitro*. Mononuclear cells (MNCs), including peripheral blood mononuclear cells (PBMCs), cord blood mononuclear cells, or bone marrow mononuclear cells, are the main sources of such cells [8, 9, 14, 15]. Therefore, it is essential to explore adequate cryopreservation methods for MNC to obtain functionally competent tolDC with tolerogenic potential. Consequently, determining the impact of cryopreservation and the ability of tolDC to stimulate Tregs in an organism with autoimmune pathology remains a relevant research objective.

The purpose of the study was to investigate the effectiveness of dendritic cells derived from cryopreserved bone marrow mononuclear cells in establishing a tolerogenic platform in recipients through the mechanism of stimulating T-regulatory cells in an experimental model of adjuvant arthritis.

Materials and Methods. The experiments were performed on CBA/H mice in accordance with the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447–IV dated 21.02.2006). Bone marrow was flushed out from the femoral bones of the mice [5]. To obtain mononuclear cells (MNCs), the cell suspension of bone marrow was centrifuged in a density gradient (1.077 g/ml) of Trazograph (Unique Pharmaceutical Laboratories, India) [5]. Cryopreservation of MNC was performed using 10 % dimethyl sulfoxide (DMSO) as a cryoprotectant at a rate of 1°C/min according to two protocols: Protocol 1 (conventionally accepted) down to -80°C, and Protocol 2 (theoretically developed by us) down to -40°C, followed by immersion in liquid nitrogen at -196°C in both cases (designated as CryoR1MNC and CryoR2MNC, respectively) [9].

Immediately after thawing and washing from the cryoprotectant, CryoR1–and CryoR2MNC were cultured for 7 days in RPMI–1640 medium with the addition of mouse recombinant GM–CSF (Sigma–Aldrich, England) and IL–4 and dexamethasone (Sigma–Aldrich, England) to obtain CryoR1DCs and CryoR2DCs, respectively [5]. DCs derived from native MNCs (NatDCs) were used as a control. The belonging of DCs to immature tolerogenic ones was confirmed by the expression of distinctive phenotypic markers on a FACS Calibur flow cytometer (Becton Dickinson, USA) using monoclonal antibodies (MAT): CD11b (FITC), CD14 (FITC), CD83 (FITC), CD80 (FITC) and CD86 (FITC) (BD Biosciences, USA) [9].

Adjuvant arthritis (AA) was induced in mice by subplantar injection of complete Freund's adjuvant (Santa Cruz, USA). The development of AA was assessed using the arthritis index (AI) [9].

DCs derived from native or CryoMNCs were intravenously administered to AA mice at a dose of 5x105 cells per mouse on the 14th day of AA development, and dexamethasone (Dex) was used as a control [5]. AI and the tolerogenic function of DCs were evaluated 7 and 14 days after DC administration by assessing their ability to induce T–regulatory cells in the spleen of animals. Phenotypic characterization of T–regulatory cells was performed using mAbs: CD4 (FITC), CD25 (PE), and FOXP3 (PE) (BD Biosciences, USA).

The ratio index (RI) of $CD4^+CD25^+$ – to $FOXP3^+$ cells in units was introduced. If this index was close to "1", as in the intact control, it indicated "harmonization" of the Treg subpopulation.

The content of *foxp3* gene transcripts was evaluated by RT–PCR in spleen cells of AA animals after tolDC administration. PCR was performed using primer pairs for the *foxp3* gene and the "housekeeping" gene *β-actin*, which were designed on the basis of the GenBank database of the National Center for Biotechnology Information (NCBI BLAST, USA).

Cell lysis, total cellular RNA obtaining, genomic DNA extraction, and cDNA amplification were performed by PCR using the Luna Cell Ready One-Step RT–qPCR Kit E3030S (Biolabtech, Ukraine). Reverse transcription and amplification were performed in an ANK–16 nucleic acid analyzer.

The relative level of gene expression (R) was calculated by the method of ΔΔCt:

$$R = \frac{(E_{target})^{\Delta cp\ target\ (intact - experiment)}}{(E_{\beta\text{-}actin})^{\Delta cp\ \beta\text{-}actin\ (intact - experiment)}},$$

where R is the relative expression level of the target gene (*foxp3*); E_{target} and $E_{\beta\text{-}actin}$ are the PCR efficiency of the target gene (*foxp3*) and *β-actin* cDNA, respectively; Δcp_{target} and $\Delta cp_{\beta\text{-}actin}$ are the deviations of Cp (intact – experimental) of *foxp3* and *β-actin* cDNA, correspondingly. The amplification efficiency for both *foxp3* and *β-actin* was 95-96 %.

Statistical data processing was performed using the SPSS software package (Statistics 17.0, USA). The number of animals in each group was n=5–7. The experimental data are presented as mean±standard deviation. The reliability of differences between groups was assessed by the Mann-Whitney method. Differences were considered statistically significant at $p<0.05$.

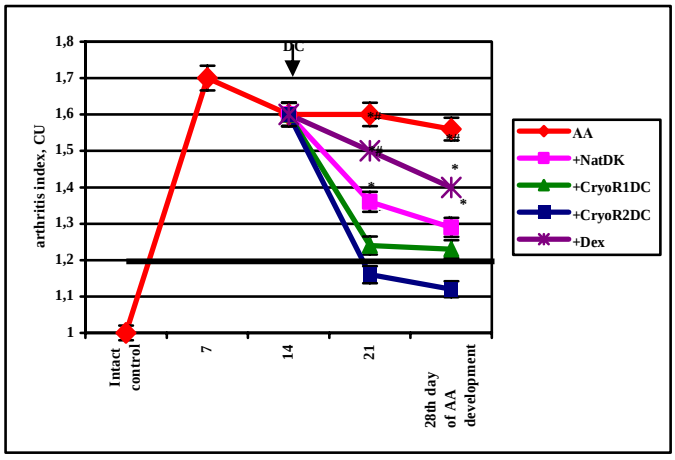


Fig. 1. Arthritis index values in AA mice before and after administration of NatDCs or CryoDCs.

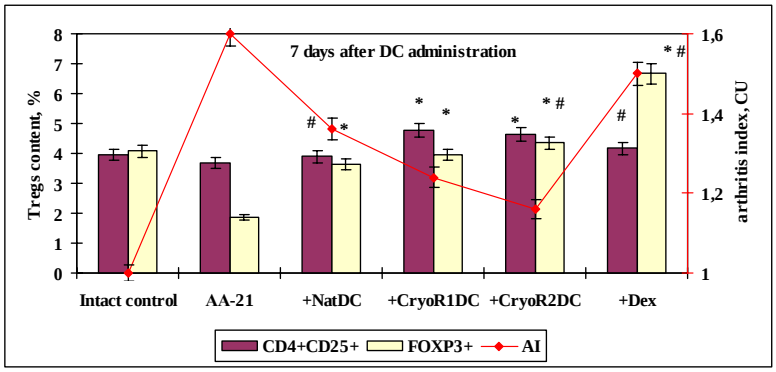
Notes: DC was administered on day 14 of AA development. IA was evaluated at 7 days (21 days of pathology development) and 14 days (28 days of pathology development) after DC administration; * – indices have statistically significant differences from the group of AA animals; # – from the group of AA+CryoR1DC animals (P<0.05); AI in intact animals (intact control) was considered as "1".

Results of the study and their discussion. As can be seen from Fig. 1, AI in animals with AA on day 14 of pathology development was 1.6±0.03 cond. units. The introduction of different types of DCs to animals with AA led to a sharp decrease in IA both after 7 and 14 days. In 7 days (21 days of pathology development) after the administration of NatDC and CryoR1DC, this indicator was 1.36±0.03 and 1.24±0.02 cond. units, respectively, and when CryoR2DC was given, it was as close as possible to the control level (1.16±0.02 and 10±0.02 cond. units, correspondingly).

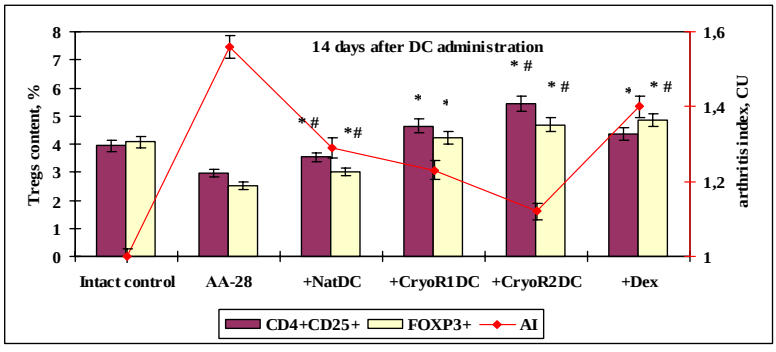
The difference between the groups in IA parameters remained 14 days (28 days of pathology development) after the administration of DCs, moreover, CryoR2DCs provided a prolonged therapeutic effect (AI – 1.12±0.02 cond. units).

Obviously, the improvement of the main clinical and diagnostic indicator – AI may be a consequence of the growth of Tregs content and functional state after adoptive therapy of recipients with AA tolDCs.

Indeed, the data displayed in Fig. 2 indicate a significant change in the quantitative and qualitative indicators of Tregs, which provide a state of immune tolerance in mammals and express the markers CD4CD25 and FOXP3 [4].



A



B

Fig. 2. The content of Treg cells ($CD4^+CD25^+$ and $FOXP3^+$) and AI before and after administration of NatDC or CryoDC to AA animals.

Notes: A – administration of DCs on day 14 of AA, evaluation after 7 days (21 days of AA development); B – administration of DCs on day 14 of AA, evaluation after 14 days (28 days of AA development); * – indices have statistically significant differences from the corresponding group of animals with AA; # – from the group of animals with AA+ CryoR1DC (P<0.05).

On day 21 of pathology development (AA-21), the AI was 1.6 times higher than the control one (Fig. 2 A). At the same time, a significant "imbalance" in the concentration relations of $CD4^+CD25^+$ and $FOXP3^+$ cells is notable. The content of $CD4^+CD25^+$ cells did not differ from the intact control, whereas the content of $FOXP3^+$ cells decreased almost twice, indicating inhibition of this protein production in AA. It should be underlined that in intact control, both assessed Tregs subpopulations are in a certain concentration balance, i.e., in a "harmonised" immunospace (IC – 1.0 cond. units). As can be seen, this state changes significantly in the conditions of AA development (IC – 1.98 units). In 7 days after DC administration, these parameters changed but to various significantly, degrees

depending on the type of DC. Thus, NatDCs almost doubled the number of $FOXP3^+$ cells and did not affect the number of $CD4^+CD25^+$ cells, which was accompanied by a decrease in AI compared to AA animals (1.36±0.03 and 1.60±0.03 cond. units, respective). It would seem that an increased content of the $FOXP3^+$ cell subpopulation in the total Tregs pool is more important for improving the clinical status of animals with AA. According to the AI, as the main evidence of the therapeutic effect, CryoP1– and CryoP2DCs prevailed over NatDCs in minimising this index. In contrast to NatDCs, two types of CryoDCs induced an augmentation of both $CD4^+CD25^+$ and $FOXP3^+$ cells, and in each case, with a boost in the growth of $CD4^+CD25^+$ cells. Thus, there is an obvious "harmonisation" of the ratio of these subpopulations when CryoR1– or CryoR2DC was administered (IC – 1.20 and 1.07 cond. units, respectively) compared to AA (IC – 1.98 cond. units). The data obtained indicate that 7 days after the administration of CryoR1DC, the AI was significantly reduced compared to the application of NatDC. Remarkably, the decrease in AI occurred against the background of a certain rise in the concentration of both Treg subpopulations. The maximum inhibition of AI was associated with CryoP2DCs, emphasising their highest immunosuppressive activity. Based on the mentioned above, a probable explanation for the greater corrective and therapeutic effect of CryoR1DCs compared to CryoR2DCs may be their increased activity to form FOXP3 protein in cells. Noteworthy, the use of Dex, as a powerful anti–inflammatory agent, had almost no effect on $CD4^+CD25^+$ cells and AI, while significantly increased the content of $FOXP3^+$ cells. This may be for several reasons. Firstly, an increase in the content of $FOXP3^+$ cells does not yet indicate their ability to implement a suppressive effect. This requires a certain concentration of this protein in the cell. It is more likely that Dex stimulates the growth of $FOXP3^+$ cells with a low concentration of this protein, which is not enough to inhibit such a severe pathology as RA and its experimental analogue, AA.

On day 28 of the pathology (AA–28), a 1.2-fold decrease in $CD4^+CD25^+$ was observed with an unchanged number of $FOXP3^+$ cells (Fig. 2 B), in contrary to these parameters on day 21 of AA development (AA–21). At the same time, AI in animals on the 21st and 28th day of pathology development did not show any difference (1.6±0.03 and 1.56±0.02 units, respectively). At 14 days after the administration of NatDC, the decrease in AI occurred against the background of a slight reduction in the content of $FOXP3^+$ cells compared to day 7 (1.29±0.02 and 1.36±0.03, correspondingly). Meanwhile, after the administration of CryoR1DC, the AI did not change, as well as the content of $CD4^+CD25^+$ and $FOXP3^+$ cells did not undergo any changes compared to these indices 7 days after administration. The therapeutic effect of CryoR2DC is striking when the period of its evaluation is prolonged (from 7 to 14 days). In contrast to CryoR1DC, CryoR2DC was able to induce the formation of $CD4^+CD25^+$- and $FOXP3^+$ cells in a "harmonised" ratio (IC – 1.16) two weeks after administration. Clearly, this contributed to the maximum reduction of the clinical manifestation of pathology in AI (1.12±0.02 cond. units) and brought it closer to the intact control (1.00±0.02 cond. units). These facts indicate that the developed mode of cryopreservation of MNCs–R2 ensures the formation of DCs with prolonged tolerogenic function.

The implementation of adaptive tolDC therapy to stimulate Tregs in ADs is a trendy area of present-day experimental and clinical immunology. Particular attention is paid to the study of the expression level of the *foxp3* gene, which controls the production of this protein and, accordingly, the suppressor activity of Tregs. The importance of these studies is rising, taking into account the use of Tregs grown from their cryopreserved precursors.

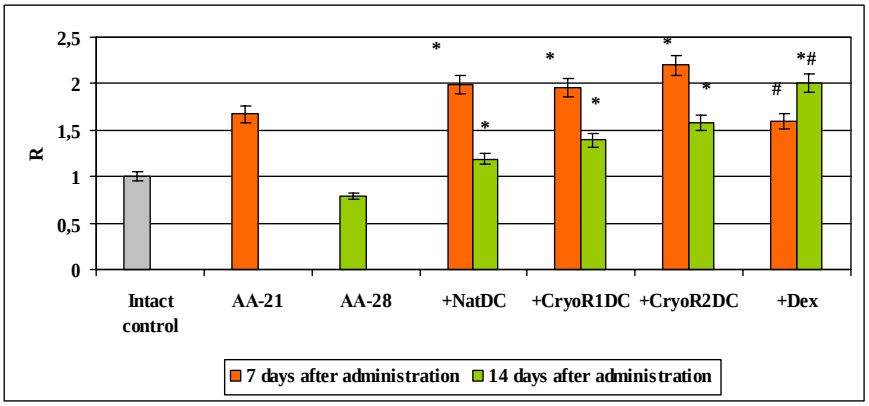


Fig. 3. Relative expression level (R) of the *foxp3* gene in spleen cells of AA animals 7 and 14 days after administration of NatDC or CryoDC. Notes: R value in the group of intact animals (intact control) is taken as 1; * – indices have statistically significant differences from the groups of AA animals; # – from the group of AA+CryoR1DC animals in relation to the corresponding time points after administration (P<0.05).

During AA development, the amount of $FOXP3^+$ cells was inversely related to the level of *foxp3* gene expression (Fig. 3) in animal spleen cells, which may be a consequence of compensation for their functional activity.

It is known that one of the evaluated Tregs markers is an intracellular protein (FOXP3), and the other (CD25) is a membrane receptor. The formation of the immunosuppressive function of Tregs is carried out through the acceptance of the cytokine IL–2 by the CD25 receptor, which is accompanied by the production of FOXP3 protein, an activator of the suppressor link of the immune response [10]. FOXP3 is the most specific marker of Tregs, the *foxp3* gene being responsible for their development and suppressive function [3]. Its high expression was found in peripheral $CD4^+CD25^+$ Tregs, but FOXP3 protein can also be expressed by effector T cells after activation [4]. Besides, it has been shown that the expression of the transcription factor FOXP3 by $CD4^+CD25^+$ T cells is not always accompanied by the acquirement of a suppressor function and a stable regulatory phenotype [4]. This is confirmed by our data on the absence of a suppressor effect of Dex–induced $FOXP3^+$ cells.

A clear correlation between the level of *foxp3* gene expression and the content of $FOXP3^+$ – Tregs is not always evident, which is consistent with the literature [4]. The authors noted that impaired immunological tolerance in ADs leads to changes in the signals of the microenvironment and cytokine network. This can negatively affect the transcriptional and epigenetic regulation of the *foxp3* gene, which is reflected in the generation and suppressor function of Tregs.

It should be noted that the level of *foxp3* gene expression 7 and 14 days after the administration of NatDC or CryoDC under different regimes was higher compared to that of intact animals and AA animals. The administration of NatDC and CryoP1DC after 7 days induced the same expression of the *foxp3* gene and production of the transcription factor (FOXP3 protein) in spleen cells of AA animals. However, as for the AI index, it was different. Thus, the clinical status of animals may be affected by other factors, such as cytokine imbalance [1, 7]. Conversely, despite the fact that the level of expression of the studied gene in the group with Dex administration at this time was lower than in other groups, the content of $FOXP3^+$ cells was quite high. As the time after DC therapy was prolonged from day 7 to day 14, the level of *foxp3* gene expression decreased in all groups except for the group of animals with Dex administration. While on day 7 the level of *foxp3* gene expression in this group did not differ from that of AA animals, on day 14, when it decreased in all groups, it rose, on the contrary. The point is that corticosteroids are powerful immunosuppressants. Epigenetic regulation of genes, primarily DNA methylation, plays a significant role in controlling their function and is involved in the expression of the Treg transcriptional regulator FOXP3 [6]. The mechanism underlying this unique epigenetic effect of glucocorticoids is associated with a decrease in the expression of the DNA methyltransferase gene, which leads to a significant inhibition of DNA methylation [3]. This causes a disruption of the gene's transcriptional activity and, as a result, can lead to an elevation of FOXP3 protein production. However, the suppressive function of Tregs induced by Dex both on day 7 and 14 after its administration did not result in a significant decrease in AI. The highest expression of the *foxp3* gene in spleen cells of animals with AA after DC therapy was induced by CryoP2DC both on day 7 and 14 after their administration. This was reflected in an increase in FOXP3 protein production in Treg cells and led to a maximum reduction in AI.

Conclusions

1. A method of cryopreservation of bone marrow mononuclear cells (R2) has been developed, which, in contrast to the conventional method (R1), provides significantly enhanced tolerogenic activity of the DCs obtained from them. They significantly increase the content of Treg cells ($CD4^+CD25^+$, $FOXP3^+$) against the background of activation of *foxp3* gene expression. The use of DCs obtained under such conditions led to a significant decrease in the clinical diagnostic indicator – AI. Moreover, such DCs had a more prolonged therapeutic effect.

2. The therapeutic effect of CryoDC was significantly higher compared to dexamethasone, which did not significantly reduce AI, although on day 14 it slightly increased the level of *foxp3* gene expression and the content of $FOXP3^+$ cells.

3. Analysis of the content of $CD4^+CD25^+$ and $FOXP3^+$ cells, the level of *foxp3* gene expression and AI on days 7 and 14 after administration of different types of tolDCs in the AA model showed that the improvement in the clinical status of animals is more correlated with the content of $FOXP3^+$ T–regulatory cells than with the level of *foxp3* gene expression.

The study of the mechanisms of improving the tolerogenic function of DCs derived from cryopreserved precursors at the cellular and molecular levels, followed by the activation of the functional potential of Treg cells, opens up the perspectives of creating fundamentally new, more effective approaches to the treatment of autoimmune diseases in clinical practice.

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