Cryomedicine, Clinical and Experimental Transplantology

https://doi.org/10.15407/cryo36.01.051
UDC 611.018.53.088.086.13:618.48:[611.018.46:612.017.1

Anatoliy M. Goltsev *, Tetyana G. Dubrava, Olena D. Lutsenko, Larysa V. Sokil, Lyudmyla G. Chernyshenko, Ihor G. Grisha
Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine
* cryopato@gmail.com

LYOPHILISED HUMAN CORD BLOOD LEUKOCONCENTRATE TRIGGERS FORMATION OF DENDRITIC CELLS WITH A TOLEROGENIC PHENOTYPE

In this research we investigated the lyophilized human cord blood leukoconcentrate (lHCBL) as an inducer of the in vitro formation of dendritic cells (DCs) with a tolerogenic phenotype, obtained from native mononuclear cells (MNCs) of the animals' bone marrow. The phenotypic affiliation of DCs to tolerogenic was assessed by the expression of markers characteristic of them: CD11b, CD80, CD86, CD14, and CD83. To form the tolerogenic phenotype of DCs, lHCBL was additionally added to the RPMI-1640 culture medium containing mouse recombinant GM-CSF, IL-4 and dexamethasone in different doses (1×10^3, 1×10^4 and 1×10^5 cells/ml). A dose-dependent effect of lHCBL on the in vitro formation of DCs with phenotypic features of tolerogenic cells from bone marrow MNCs was established. The conditions for obtaining DCs in vitro under the influence of LHCBL with the maximum tolerogenic effect were determined, that was manifested by a significant decrease in the expression level of co-stimulatory molecules CD80 and CD86 (by 1.6 times), an increase in CD11b (by 1.2 times) and the content of $CD11b^+$ DCs (by 25%) compared to the control values, that confirms the tolerogenic phenotype of the obtained cells. This fact indicates a limitation of the ability of DCs formed in culture to implement cooperative interactions and activation of T-effector cells.

Key words: *lyophilized human cord blood leukoconcentrate, dendritic cells, tolerogenic phenotype.*

Currently, the issue of treating autoimmune diseases (AIDs) remains relevant. Recently, tolerogenic dendritic cells (tolDCs) have been used to overcome such diseases. At the same time, an attention is focused both on improving the methods of obtaining tolDCs from immature precursors and on the ways to increase their tolerogen-inducing potential. Existing approaches to generating tolDCs are based on the use of multicomponent inducers of their formation *ex vivo*. Classical inducers of immune tolerance formation of dendritic cells (DCs) are granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-10 (IL-10), transforming growth factor β (TGF-β), prostaglandin E2, histamine, vitamin D3 and its analogues, glucosamine, cobalt protoporphyrin, *etc.* [4, 20, 25, 39]. The results of using these reagents demonstrated high stability of *in vitro* DCs to maturation and secretion of increased levels of anti-inflammatory IL-10 [22]. In addition, various drugs with immunosuppressive activity are used to modulate the differentiation and functional activity of tolDCs, namely: corticosteroids, cyclosporine, tacrolimus, rapamycin, aspirin, deoxypergualin [27]. These compounds prevent DC maturation and/or their activation, they are also able to reduce IL-12 production, induce the formation of contact-dependent regulatory T cells (Treg) [4, 29].

Reference: Goltsev AM, Dubrava TG, Lutsenko OD, Sokil LV, Chernyshenko LG, Grisha IG. Lyophilised human cord blood leukoconcentrate triggers formation of dendritic cells with a tolerogenic phenotype. *Probl Cryobiol Cryomed*. 2026; 36(1): 51—7. https://doi.org/10.15407/cryo36.01.051

For the induction of tolDC formation, it is also advisable to use human cord blood (HCB) components. HCB is recognized as a multicomponent biosystem, which, along with the cell component, contains such plasma elements as IL-10, human chorionic gonadotropin (hCG), estradiol, progesterone, cortisol [7]. These compounds have a tolerogen-inducing effect on the mother's immune competent cells, meaning they prevent rejection of a fetus carrying paternal antigens.

Almost 30 years ago, it was established that under the influence of IL-10, immature DCs acquired phenotypic and functional properties inherent to tolDCs [34]. The IL-10-modulated human DCs are known to be characterized by stable increased tolerogenic activity, which makes their clinical use possible [16]. In the formation of immune system tolerance, hCG plays an important role, being one of the hormones secreted in the pregnancy first trimester [30]. During pregnancy, hCG generates the formation of tolDCs by activating the expression of indoleamine-2,3-dioxygenase, which leads to tryptophan depletion and the cessation of T-cell proliferation and anergy [13]. The ability of hCG to modify the immune activation of T-cells by inhibiting the production of IL-2 has also been found [13]. Similar effects have been reported with maternal serum, cord blood serum, and placental extract [30]. In a mouse model, adoptive transfer of DCs treated with hCG before mating was protective for pregnancy stability, accompanied by increased Treg numbers and TRF-β and IL-10 expression [3]. The effect of hCG on the tolerogenic properties of mouse bone marrow-derived DCs was also confirmed by increased IL-10 production and inhibition of antigen-specific T-cell proliferation *in vitro* [3]. The described effect indicates the contribution of hCG to maternal-fetal tolerance during pregnancy by modifying DCs towards a tolerogenic phenotype [35, 36]. Other hormones (estradiol, progesterone, cortisone), the levels of which increase in the HCB plasma during pregnancy, also induce the formation of DCs with a tolerogenic phenotype [21, 33].

It is known that in HCB, along with hormones that induce the tolerogenic phenotype of DCs, cells with suppressor activity are present [7, 9]. It has been shown that the HCB leukoconcentrate (HCBL) contains Treg that are able to secrete anti-inflammatory IL-10 and block the immune response of T-effector cells under conditions of chronic graft-versus-host disease [31] or in animals with atopic dermatitis [15].

The demand for HCB in clinical practice necessitates the establishment of its stocks, therefore, the development of technologies for its storing at ultralow temperatures (–80, –196 °C) [1] is relevant. It is the very those conditions that ensure the preservation of structural and functional properties of cells of different levels of differentiation and the content of regulatory mediators in HCB [6]. It should be noted that the implementation of technologies for long-term storage of HCB and its components at ultralow temperatures requires significant financial costs, therefore, an alternative approach may be lyophilization [24]. It is important that lyophilized biological material in general, and HCBL in particular, unlike cryopreserved one, can be stored in household refrigerators at low temperatures. Moreover, such conditions are more optimal for transporting such material. In a rat model of atopic dermatitis, which is an autoimmune pathology, clinical signs have shown a "delayed" manifestation of the therapeutic effect of cryopreserved HCBL compared to lyophilized (lHCBL) [14]. This fact can be explained by the increase of the suppressive activity of lHCBL due to the influence of biologically active substances, in particular hormones, with tolerogen-inducing potential, which are additionally released during the lyophilization process.

Therefore, the properties of lHCBL that can affect the formation of the *in vitro* tolerogenic phenotype of DCs obtained from native autologous precursors are a prerequisite for its use in the treatment of pathologies of autoimmune genesis.

Based on the above, the aim of this work was to determine the features of the influence of lyophilized human cord blood leukoconcentrate on the nature of the formation of phenotypic signs of dendritic cells derived from native animal bone marrow mononuclear cells.

MATERIALS AND METHODS

The research was performed in 20-week-old CBA/H mice weighing 24–26 g. Experimental animals were provided by the Biomodelservice enterprise (Kyiv, Ukraine) with permission for further breeding under standard conditions at the vivarium of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (IPCC of the National Academy of Sciences of Ukraine, Kharkiv). All manipulations with animals were approved by the Bioethics Committee

of the IPCC of the National Academy of Sciences of Ukraine (protocol No. 5 dated November 26, 2019) in accordance with the main provisions of the Law of Ukraine"On the Protection of Animals Against Cruelty" (No. 3447-IV dated February 21, 2006), the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986). Human cord blood was collected with the informed consent of the mother immediately after the birth of the child. The HCB leukoconcentrate in autoplasma was obtained by sedimentation of erythrocytes after adding polyglucin to the cord blood (Yuria-Pharm, Ukraine). The leukoconcentrate was poured into sterile penicillin vials of 1 ml, which were placed on the shelf of the UZV-2 sublimation unit (Special Designing and Technical Bureau at the IPCC of the National Academy of Sciences of Ukraine) and lyophilized as reported by A.M. Goltsev *et al*. [10]. The lHCBL samples were stored at 4 °C. The lHCBL was rehydrated by adding 1 ml of physiological solution to the vials (Yuria-Pharm) for 10 min and carefully mixed to prevent foaming.

Bone marrow cells from femurs of CBA/H mice were obtained by washing with RPMI-1640 (Biowest, France) supplemented with 3% fetal calf serum (Biowest) and 2% sodium citrate (Sigma-Aldrich, USA) (hereinafter referred to as handling medium). The bone marrow cell suspension was passed through a nylon filter with a pore diameter of 100 μm (Falkon, USA), centrifuged at 200g for 10 min, and the resulting pellet was re-suspended in handling medium. Mononuclear cells (MNCs) from the bone marrow suspension were obtained by density gradient centrifugation (1.077 g/ml) of the drug "Trazograf 76%" ("Unique Pharmaceutical Laboratories", India) [11].

The MNCs to obtain DCs were cultured immediately after their isolation from the bone marrow in plastic Petri dishes with a diameter of 3 cm in RPMI-1640 with the addition of 10% fetal calf serum and 1% antibiotic solution (100 units/ml penicillin, 0.1 mg/ml streptomycin), at a concentration of 3—5 × 10^6 cells/ml. The cells were cultured at 37 °C in a 5% CO_2 atmosphere. After 2 hrs, the medium with non-adherent cells was removed, and RPMI-1640, containing murine recombinant GM-CSF (20 ng/ml), IL-4 (5 ng/ml) and dexamethasone (0.4 мg/ml) (Sigma-Aldrich, UK) was added to the monocytes (adhesive fraction of MNCs) [8, 38]. After 3—4 days, a fresh portion of culture medium was added with different doses of lHCBL: 1 × 10^3 (1), 1 × 10^4 (2) and 1 × 10^5 (3) cells/ml of medium (lHCBL 1, 2, 3). On day 7 the DCs were collected for further cytofluorimetric analysis of their phenotypic affiliation to toleroge-nic ones.

The phenotypic affiliation of DCs to tolerogenic ones was assessed by the expression of characteristic phenotypic markers on a flow cytometer "FACS Calibur" (Becton Dickinson, USA) using anti-mouse monoclonal antibodies: CD11b FITC, CD14 FITC, CD83 PE, CD80 FITC and CD86 FITC (BD Biosciences, USA). An additional index for assessing the expression of these membrane markers of immune competent cells derived from MNCs was the average fluorescence intensity (AFI), which reflects the density of expression of a certain marker on the cell. In addition, the total fluorescence index (TFI) was assessed, which is an integral parameter of the expression of these marker molecules. To calculate it, the number of cells expressing the marker was multiplied by the AFI.

Statistical data processing was performed using the "SPSS Statistics 17.0" software (USA). The number of animals in each group was 5. The results were analyzed using nonparametric analysis of variance using the Kruskal-Wallis test (H-test) with subsequent assessment of the significance of differences between groups using the Mann-Whitney test (U-test). Differences were considered significant at $p < 0.05$. The analysis was performed with the calculation of the median (Me) and the 25 and 75^{th} percentiles.

RESULTS AND DISCUSSION

The implementation of the functional potential of DCs depends on the structural organization and functional activity of these cells [8, 23, 37]. In this case, the expression rate of surface membrane costimulation molecules — CD80/CD86, which belong to immunoglobulin receptors, is of significant importance. Their ligands are, respectively, CD28 and CTLA-4 molecules expressed on T lymphocytes. Contact of CD80/CD86 with these ligands ensures the activation and proliferation of T cells. On the contrary, the low level of expression of CD80 and CD86 molecules on DCs is an important sign of the DCs becoming tolerogenic, namely: suppressive activity towards T effectors and stimulatory activity towards Treg [7]. The transition of MNCs to immature tolDCs can be assessed (in addition to functional potential) by certain immune phenotypic features.

As a rule, tolDCs include cells with a low level of expression of both major histocompatibility complex class II molecules and co-stimulatory molecules CD80/CD86 and a minimal level of expression of the CD83 marker, as well as an increased level of CD11b [11, 23].

The results of flow cytometer analysis obtained by us show that on day 7 of *in vitro* cultivation of DCs from native MNCs (control) cells characteristic were formed, among which 57.2% expressed the CD80 marker and 56.0% — the CD86 marker (Table). The addition of lHCBL to the culture medium changed both the percentage of formed DCs and the level of expression of the indicated costimulatory molecules on their membrane. At the same time, a clear dose-dependent effect of lHCBL was observed. Thus, the maximum and almost identical decrease in the concentration of $CD80^+$– and $CD86^+$– cells (by 2 and 1.7 times, respectively, compared with the control) occurred after the addition of lHCBL 2 to the culture.

The functional potential of immune competent cells is known to depend on the density of expression of certain receptors on their surface membrane [17]. Based on this fact, we assessed the TFI of the studied molecules on DCs. It was found that after the addition of lHCBL2 to the DC culture, the TFI of CD80 and CD86 molecules decreased equally, namely: by 1.6 times compared to the control. Therefore, at the minimum level of expression of the mentioned markers after the addition of lHCBL, the possibility of the DCs formed in the

Expression of phenotypic markers of DCs formed *in vitro* from MNCs under the conditions of the addition of lHCBL; Me (LQ; UQ)

DCs, derived from native MNCs	Parameter	$CD11b^+$	$CD80^+$	$CD86^+$	$CD14^-CD83^+$
Control (no lHCBL added)	Content,%	56.4 (52.5; 61.4)	57.2 (52.4; 63.9)	56.0 (54.4; 59.3)	0.4 (0.3; 0.5)
	AFI, arb.units	761.0 (738.2;787.4)	520.3 (518.2;531.5)	491.6 (446.7;537.4)	884.6 (835.54;935.2)
	TFI, arb.units	42920.4 (38733.9; 47544.6)	28944.9 (26880.2; 34084.9)	29127.3 (22889.5; 30095.5)	353.84 (288.7; 407.6)
lHCBL1	Content ,%	59.3 (55.5; 61.2)	44.5 (41.2; 48.3) *	44.1 (38.8; 49.6) *	0.2 (0.1;0.3) *
	AFI, arb.units	770.0 (753.2; 802.6)	518.6 (487.1; 535.8)	453.1 (415.3; 491.8)	963.1 (893.5; 1027.4) *
	TFI, arb.units	45661.0 (42736.3; 46124.1)	23077.7 (22200.2; 24984.9) *	19982.6 (18465.6; 20582.8)	232.9 (105.9; 294.8) *
lHCBL 2	Content,%	70.4 (68.7; 74.4) *	27.9 (25.4; 31.6) *	33.1 (31.5; 35.8) *	0.1 (0.1; 0.2) *
	AFI, arb.units	732.9 (697.8;792.5)	609.8 (576.9; 644.9) *	539.0 (498.8; 546.1) *	1137.6 (1068.4; 1232.7) *
	TFI, arb.units	51584.3 (49306.3; 54465.0) *	18012.9 (13667.3; 19237.9) *	17864.0 (15058.8; 19539.8) *	113.8 (104.5; 120.3) *
lHCBL 3	Content ,%	55.7 (53.2; 56.9)	50.8 (46.8; 53.4)	48.7 (45.4; 52.5)	1.5 * (1.4; 1.7)
	AFI, arb.units	812.5 (783.9;839.6)	461.8 (421.5; 511.3)*	460.7 (459.7;472.2)	992.8 (946.5; 1023.6) *
	TFI, arb.units	46780.8 (40771.1; 48106.8)	23916.3 (21125.1; 26622.2) *	22984.3 (20766.0; 24170.3)	1438.6 (1380.0; 1770.8) *

* Changes are significant *vs* the control; ($p < 0.05$ on the Mann-Whitney test).

culture to implement cooperative interactions and activate T-effector cells is limited, which is a confirmation of their tolerogenic phenotype.

The multifunctional potential of HCB cells necessitates the certification of possible side effects, in particular the formation of antipodal tolerogenic mature DCs with the $CD14^{-}CD83^{+}$ phenotype. As evidenced by the findings, after complementing the culture with lHCBL1 or lHCBL2, a dose-dependent decrease in the content of DCs with a certain phenotype was observed by 2.0 and 4.0 times, respectively, compared with the control (0.4%). Moreover, the TFI, as an integral index of the expression rate of the CD83 marker, also decreased significantly, and after the addition of LHCBL 2 to the greatest extent. Against the background of such changes, it is interesting that after the use of lHCBL3, the content of $CD14^{-}CD83^{+}$ DCs increased by 3.8 times compared with the control. This indicates the presence in lHCBL of certain compounds and plasma protein structures, which, when their content is critically increased, can induce the formation of mature DCs. Thus, despite the "minimal" immunogenic potential [5, 18], lHCBL can, at least at the interspecies level, act as an activator of the immune response. In addition, an alternative process may be spontaneous differentiation of DCs under conditions of increased density of intercellular contacts and "disharmony" of interaction of signaling molecules, for example, the SWAP-70 protein (the switch-associated protein 70), associated with the switch [26]. Indeed, according to M. Bros *et al.* [2] and C. Ocana-Morgner *et al.* [26], the absence of SWAP-70 protein expression was accompanied by spontaneous maturation of DCs in the spleen and bone marrow of animals, which can be explained by an increase in the level of expression of the CD83 maturity marker on DCs after the addition of lHCBL to the culture medium 3.

The tolDCs are known to be characterized with the expression of the surface membrane molecule CD11b, with the participation of which they inhibit T-cell activation [12, 19]. This molecule belongs to the superfamily of integrin heterodimeric receptors, which include б (CD11a, CD11b, CD11c and CD11d) and в (CD18) subunits [32]. The "repertoire" of the formation of functionally complete receptors, regulating the balance between the immune or tolerogenic activity of DCs depends on their various combinations and expression density. Thus, against the background of cooperation of CD11b and CD18 subunits, the Mac1 receptor is formed on DCs, which causes inhibition of their interaction with T-cells [32]. The implementation of this effect is manifested by a decrease in the level of expression of the costimulatory molecule CD86 on DCs and, as a consequence, the absence of binding of the Mac1 receptor to its ligand ICAM-1 on the surface of the T-lymphocyte [28].

According to the results of flow cytometry analysis, it was found that the content of $CD11b^{+}$ DCs formed *in vitro* from native MNCs was 56.4%, and their CPS was 42,920.4 conventional units (Table). In addition, it should be noted a significant dose-dependent effect of the effect of lHCBL on the quantitative and qualitative indices of $CD11b^{+}$ cells. Thus, the addition of lHCBL1 or lHCBL3 to the culture medium did not significantly affect the content of $CD11b^{+}$ DCs and the expression rate of this marker compared to the control. However, after the addition of lHCBL 2, both an increase in the content of $CD11b^{+}$ DCs by 25% and a 1.2-fold increase in the expression rate of this marker (TFI) *versus* the control values were observed.

Thus, a clear dose-dependent effect of the action of lHCBL on immune competent cells was established, which can cause both the development of pathological and maintenance of physiological conditions. The findings indicate that under the influence of lHCBL2 *in vitro*, tolDCs were formed with a lower expression rate of co-stimulatory molecules CD80 and CD86 and a maximum degree of the one for the CD11b molecule. The tolDCs obtained in this way can be used in the treatment of autoimmune diseases.

CONCLUSIONS

A dose-dependent effect of the lHCBL on the *in vitro* formation of DCs with phenotypic signs of tolerogenicity from bone marrow MNCs was established.

The conditions for obtaining DCs *in vitro* under the influence of lHCBL with the maximum tolerogen-inducing effect were determined, that was manifested by a significant decrease in the expression rate of co-stimulatory molecules CD80 and CD86 (by 1.6 times), an increase in CD11b (by 1.2 times) and the content of $CD11b^{+}$ DCs (by 25%) compared to control values, that confirms the tolerogenic phenotype of the obtained cells.

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Received 03.10.2025
Accepted for publication 23.02.2026

*А.М. Гольцев *, Т.Г. Дубрава, О.Д. Луценко, Л.В. Сокіл, Л.Г. Чернишенко, І.Г. Гриша*
Інститут проблем кріобіології і кріомедицини НАН України, м. Харків, Україна
*cryopato@gmail.com

ВИКОРИСТАННЯ ЛІОФІЛІЗОВАНОГО ЛЕЙКОКОНЦЕНТРАТУ КОРДОВОЇ КРОВІ ЛЮДИНИ ЯК ТРИГЕРА ФОРМУВАННЯ ДЕНДРИТНИХ КЛІТИН З ТОЛЕРОГЕННИМ ФЕНОТИПОМ

У роботі досліджено ліофілізований лейкоконцентрат кордової крові людини (лЛККЛ) у якості індуктора формування *in vitro* дендритних клітин (ДК) з толерогенним фенотипом, отриманих із нативних мононуклеарів (МНК) кісткового мозку тварин. Фенотипову належність ДК до толерогенних оцінювали за експресією характерних для них маркерів: CD11b, CD80, CD86, CD14, CD83. Для формування толерогенного фенотипу ДК у культуральне середовище RPMI-1640, яке містило мишачі рекомбінантні ГМ-КСФ, ІЛ-4 та дексаметозон, додатково додавали лЛККЛ у різних дозах (1×10^3, 1×10^4 та 1×10^5 кл/мл). Встановлено дозозалежний ефект лЛККЛ на формування *in vitro* з МНК кісткового мозку ДК з фенотиповими ознаками толерогенних клітин. Визначено умови отримання ДК *in vitro* під впливом лЛККЛ з максимальним толерогеніндукуючим ефектом, що маніфестувалося значним зниженням ступеня експресії костимулюючих молекул CD80 і CD86 (у 1,6 рази), підвищенням CD11b (у 1,2 рази) та вмісту CD11b$^+$ ДК (на 25 %) порівняно з контрольними показниками, що підтверджує толерогенний фенотип отриманих клітин. Цей факт свідчить про обмеження можливості сформованих у культурі ДК реалізувати кооперативні взаємодії та активацію Т-ефекторних клітин.

Ключові слова: ліофілізований лейкоконцентрат кордової крові людини, дендритні клітини, толерогенний фенотип.