IN VITRO ASSESSMENT OF IMMUNOGENICITY IN CHONDROCYTES OBTAINED FROM THE B2M KNOCKOUT INDUCED PLURIPOTENT STEM CELLS

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Today, the cell-based technologies are one of the instruments used for the cartilage tissue repair. Creation of a universal hypoimmunogenic cartilage tissue graft from the differentiated derivatives of induced pluripotent stem cells (iPSCs) might solve the problem of the lack of the cartilage cell product. However, currently there is little data on immunogenicity of such tissue-engineered preparations. The study was aimed to create a cartilage implant from the differentiated derivatives of the B2M-deficient iPSCs and assess its immunogenicity. The previously developed protocol was used to ensure differentiation of both wild-type and B2M knockout iPSCs into chondrocyte-like cells. After quality control of the resulting cell lines by conducting polymerase chain reaction and immunocytochemical assessment, the resulting cell lines were co-cultured with the peripheral blood mononuclear cells of a healthy donor. When co-cultivation was over, activation and degranulation of $CD8^+$ T cells was assessed by flow cytometry analysis based on the CD69 and CD107a expression on the cell surface, respectively. The iPSC-derived chondrocytes expressed the cartilage tissue markers. Flow cytometry analysis revealed no substantial differences in immunogenicity between the derivatives of wild-type and B2M knockout iPSCs, as well as from the cartilage tissue cells of a healthy donor. Immunogenicity of chondrocyte-like cells was higher than that of hypoimmunogenic non-edited iPSCs. The B2M knockout iPSCs demonstrated a trend towards greater activation of $CD8^+$ T cells. Thus, the B2M knockout in the iPSC-derived chondrocytes had no significant effect on the tissue immunogenicity. It is necessary to further edit the genes encoding MHC II and CD47 to obtain a less immunogenic product.

Keywords: iPSCs, regenerative medicine, chondrogenesis, chondrocytes, immunogenicity

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IN VITRO ОЦЕНКА ИММУНОГЕННОСТИ ХОНДРОЦИТОВ, ПОЛУЧЕННЫХ ИЗ ИНДУЦИРОВАННЫХ ПЛЮРИПОТЕНТНЫХ СТВОЛОВЫХ КЛЕТОК С НОКАУТОМ B2M

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В настоящее время клеточные технологии являются одним из инструментов по восстановлению хрящевой ткани. Создание универсального гипоиммуногенного трансплантата хрящевой ткани из дифференцированных производных индуцированных плюрипотентных стволовых клеток (ИПСК) могло бы решить проблему нехватки хрящевого клеточного продукта. Однако на сегодняшний день мало данных об иммуногенности таких тканеинженерных препаратов. Целью работы было создать хрящевой имплант из дифференцированных производных ИПСК, дефицитных по B2M, и оценить его иммуногенность. С помощью ранее разработанного протокола дифференцировали ИПСК как дикого типа, так и с нокаутом B2M в хондроцитарные производные. После проверки качества полученных линий методом полимеразной цепной реакции и иммуноцитохимическим исследованием кокультивировали полученные линии с мононуклеарными клетками периферической крови здорового донора. По окончании кокультивации методом проточной цитометрии оценивали активацию и дегрануляцию $CD8^+$-Т-лимфоцитов по экспрессии CD69 и CD107a на поверхности клеток соответственно. Хондроцитарные производные ИПСК экспрессировали маркеры хрящевой ткани. Цитометрический анализ не выявил существенных различий между иммуногенностью хондроцитарных производных ИПСК с нокаутом и без нокаута B2M, а также клетками хрящевой ткани здорового донора. Иммуногенность хондроцитарных производных была выше, чем у гипоиммуногенных нередактированных ИПСК. Нокаутированные по B2M ИПСК демонстрировали тенденцию к большей активации $CD8^+$-Т-лимфоцитов. Таким образом, нокаут B2M в хондроцитарных производных ИПСК не оказал существенного влияния на иммуногенность ткани. Необходимо дополнительное редактирование генов, кодирующих MHC II и CD47, для получения менее иммуногенного продукта.

Ключевые слова: ИПСК, регенеративная медицина, хондрогенез, хондроциты, иммуногенность

Финансирование: получение хондроцитарных производных из ИПСК выполнено в рамках государственного задания №122032300191-2 «Органоид-22». Иммуноцитохимический и ПЦР-анализы экспрессии хондрогенных маркеров в хондроцитарных проиводных ИПСК, а также оценка иммуногенности этих хондроцитарных производных проводились в рамках проекта РНФ #22-15-00250 «Сравнение хондрогенного потенциала хрящевой ткани, полученной с помощью первичных культур хондроцитов и дифференцированных производных индуцированных плюрипотентных стволовых клеток».

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Restoration of cartilage tissue using the cell-based and tissue-engineered products is an urgent medical task due to high prevalence of inflammatory degenerative diseases involving the surface layers of bone and cartilage tissues. Gonarthrosis, the cartilage tissue disorder, is most prevalent all over the world. This multifactorial degenerative dystrophic disease is characterized by involvement of the articular cartilage, subchondral and metaphyseal bone, as well as the synovial membrane, ligaments, capsule, and muscles, and is associated with the emergence of osteochondral masses manifesting themselves in the joints experiencing pain and a limited range of motion. The annual incidence of gonarthrosis in Russia is about 80,000 cases. According to the epidemiological research data, 8–20% of the adult population suffer from this disorder, depending on the region [1].

Gonarthrosis progression is observed year by year; different treatment approaches are used at different disease stages. According to domestic clinical guidelines, noninvasive therapy combining pharmacological and physiotherapeutic methods is recommended at early stages of the disease [2]. It should be noted that the pharmacological component of therapy involving the use of non-steroidal anti-inflammatory drugs (NSAIDs) and steroids (short course) is symptomatic and has almost no effect on the hyaline cartilage regeneration and functional activity. At the later disease stages, when noninvasive treatment is ineffective and the cartilage tissue damage is significant, there is a need for surgical intervention including abrasion chondroplasty, corrective osteotomy, and joint replacement [3].

The use of cell products for the damaged cartilage tissue repair has become a new direction of regenerative medicine. In such cases, mesenchymal stem cells (MSCs), autologous and allogeneic chondrocytes can be the sources of cells.

Despite the fact that the cell products based on autologous chondrocytes for cartilage tissue repair are already present in the medical and biotechnology market, such products have a number of limitations. First, so far these are aimed mainly at adjusting minor articular cartilage damage [4, 5]. Second, for a number of reasons, it is often impossible to obtain enough autologous chondrocytes to create a full-fledged cartilage implant [2]. Third, in individuals with some cartilage tissue disorders, autologous cell material yields chondrocytes with decreased function, which results in the need for long-term cell cultivation aimed at accumulating the required amount of material [6, 7]. In this regard, induced pluripotent stem cells (iPSCs) represent a promising and capacious source of chondrocyte-like cells, however, the data on their safety and capability of forming the full-fledged functional cartilage tissue are rather limited, while the data on immunogenicity are controversial [8, 9]. Moreover, obtaining iPSCs, their further differentiation, creation of the graft is a lengthy and expensive process that requires optimization and standardization. The literature contains information about successful engraftment of the iPSC-derived chondrocytes in the monkey articular cartilage. However, the cartilage tissue defect was small, therefore, further research involving other models is required. As for immunogenicity assessment, transplantation into cartilage defects caused no immune response in macaques, while the organoid transplanted into the osteochondral defects remained intact, but was surrounded by a large number of $CD3^+$ T cells [10].

Creation and the use of cartilage grafts based on allogenic iPSCs differentiated into chondrocytes showing decreased immunogenicity, which are universal for all recipients, seem to represent a promising approach. A number of studies demonstrate successful creation of hypoimmunogenic grafts based on the MHC knockout iPSCs [11, 12]. It has been shown that the allogenic cartilage graft obtained from the B2M knockout iPSCs contributes to accumulation of natural killers (NK cells) around the osteochondral defect *in vivo* [13]. However, such data on human iPSC-derived chondrocytes have yet to be obtained.

The study was aimed to compare immunogenicity of the samples of tissue-engineered cartilage preparations obtained *in vitro* from various cell sources.

METHODS

Cultivation of wild-type and B2M knockout iPSC lines

The iPSCs (hereinafter, iPS) were cultured as previously reported [14]. The B2M knockout iPS cell line (iPS_dB2M) was obtained earlier [15].

iPSC differentiation into chondrocyte-like cells

The thawed iPS and iPS_dB2M (used to obtain wild-type (iCh) and B2M knockout (iCh_dB2M) chondrocyte-like cells, respectively) were seeded into 6-well plates pre-treated with Matrigel (Corning; USA), in the mTeSR1 medium (STEMCELL Technologies; Canada). These were cultured in the CO_2 incubators at +37 °C and 5% CO_2 until a monolayer was formed. All further incubations were also carried out in the CO_2 incubators at +37 °C and 5% CO_2. The culture was transferred into the DMEM/F12 medium (Gibco; USA) supplemented with 10% FBS (HiMedia; India), 1% GlutaMAX (Gibco; USA), 1% penicillin/streptomycin (PanEco; Russia), 10 μM Chir (Miltenyi Biotec; USA), 10 nM retinoic acid (Miltenyi Biotec; USA). It was cultured for two days. Then the medium was replaced with DMEM/F12 with 10% FBS, 1% GlutaMax, 1% penicillin/streptomycin, 10 ng/mL TGFβ (Miltenyi Biotec; USA), 10 ng/mL BMP2 (Miltenyi Biotec; USA), B27 (Miltenyi Biotec; USA), 10 μM ascorbic acid (Sigma; USA), 1% insulin-transferrin-selenite (PanEco; Russia). This was cultured for two weeks. Then the cells were transferred to two wells on the 6-well plate. After differentiation, the cells were cultured in the DMEM/F12 medium supplemented with 10% FBS, 1% GlutaMax, 1% penicillin/streptomycin, 10 ng/mL TGFβ, 10 ng/mL BMP2.

Quality control of the iPSC-derived chondrocytes. Immunocytochemistry assessment

The monolayer cultures fixed in the 4% paraformaldehyde (PFA) were treated with the 0.1% Triton-X100 solution: for 20 min to ensure staining for a nuclear marker, for 10 min to ensure staining for surface and cytoplasmic markers. After permeabilization the cultures were treated with the blocking solution based on the 0.01M PBS with 3% goat serum and 0.1% Tween for 30 min.

The monolayer cultures were stained with primary antibodies against the Sox 9, nuclear marker of chondrogenesis (Rabbit, 1 : 400; Invitrogen, USA), aggrecan, the proteoglycan cartilage extracellular matrix marker (Mouse, 1 : 500; Invitrogen, USA), type II collagen, the marker of the fibrillar extracellular matrix of hyaline cartilage (Rabbit, 1 : 200; Abcam, UK), and type I collagen, the marker of fibrous cartilage (Rabbit, 1:800, Invitrogen, USA). Staining involving the use of primary antibody solutions based on the blocking solution was performed for 1.5 h at room temperature. Then the cultures were triple washed with 0.01 M PBS.

Alexa Fluor 555 (Goat, Anti-Rabbit, 1 : 500) and Alexa Fluor 546 (Goat, Anti-Human, 1:500) (Invitrogen; USA) were used for

Table. Primers used in the study

Gene	Primers used in the study 5'→3'	Product size, bp
SOX9	F: GAAGTCGGTGAAGAACGGGC R: CACGTCGCGGAAGTCGATAG	283
ACAN	F: AGGAGTCCCTGACCTGGTTT R: CCTGACAGATCTGCCTCTCC	167
COL1A2	F: AGGGTGAGACAGGCGAACA R: CCGTTGAGTCCATCTTTGC	184
COL2A1	F: TGGACGCCATGAAGGTTTTCT R: CCATTGATGGTTTCTCCAAACC	142
YWHAZ	F: ACTTTTGGTACATTGTGGCTTCAA R: CCGCCAGGACAAACCAGTAT	94

staining with secondary antibodies. Staining was performed for 1 h in the dark. Then the cultures were triple washed with 0.01 M PBS. The 100 ng/mL DAPI (Sigma Aldrich; USA) was used to ensure staining of the nuclei. Staining was performed for 15 min, then the cultures were triple washed with 0.01 M PBS.

The stained preparations were assessed using the Olympus IX53F fluorescent microscope with four fluorescence filters (Olympus; Japan); morphometry involved the use of the Olumpus cellSens Standard software (Olympus; Japan).

Polymerase chain reaction

To perform PCR analysis of expression, one million of cells were lysed in the RLT buffer (QIAGEN; Germany). RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN; Germany) in accordance with the manufacturer's protocol. The total RNA concentration in the sample was measured using the Infinite 200 Pro microplate reader (Tecan; Switzerland) and the I-control software. The MMLV RT kit (Evrogen; Russia) was used for synthesis of the first cDNA strand from an RNA template. Synthesis was performed according to the manufacturer's protocol. To perform real time PCR, we added 5 μL of 5x qPCRmix-HS SYBR (Evrogen; Russia), 0.8 μL of the 10 μM primer (Table 1), 18.2 μL of water, and 1 μL of template cDNA per well of the 96-well plate (SSIbio, Scientific Specialities; USA). Another 1 μL of water was added to the control wells instead of template cDNA.

The reaction was carried out using the 1000 CFX Manager version C10000 Touch thermal cycler for nucleic acid amplification (Bio-Rad; USA) and the CFX Manager software. The number of cycles was 39. The results were analyzed in Microsoft Excel (Microsoft; USA) by the ΔΔCt method.

Obtaining the primary chondrocyte culture from the donor material

Chondrocytes (hereinafter, Chondro) were isolated from the patient's biopsy (surgical) specimen. The cartilage was washed with 15 mL of DMEM with 2% penicillin/streptomycin (PanEco; Russia). Then the cartilage was placed in the clean Petri dish and chopped with the sterile scissors and scalpel in 4 mL of DMEM with 2% penicillin/streptomycin. It was washed once with the same medium in a 15 mL test tube. The cartilage pieces were incubated for 40 min on a shaker at +37 °C and 5% CO_2 in 10 mL of DMEM with 2% penicillin/streptomycin, collagenase IV (Gibco; USA), and the collagenase enzyme preparation (BioPreparat; Russia) having a concentration of 3000 U/mL.

After incubation the cartilage pieces were centrifuged for 5 min at 200 g, once washed, added 10 mL of the culture medium (DMEM/F12 with 20% FBS, 1% GlutaMax, 1% penicillin/streptomycin), and transferred to the T-75 culture flask pre-treated with the 0.1% gelatin solution. The cartilage pieces were cultured at +37 °C and 5% CO_2 until the chondrocyte monolayer was formed. The medium was changed every three days.

Obtaining the primary fibroblast culture from the donor material

The patient underwent skin biopsy of the forearm. The biopsy specimen was put in the droplet of medium (DMEM (PanEco; Russia) supplemented with 10% FBS and 1% penicillin/streptomycin) on the Petri dish and cut into small pieces (of about 1 mm) with a sharp sterile scalpel. The resulting pieces were places in different 35 mm Petri dishes in 3 mL of the culture medium and pressed by the sterile cover glass (Menzel Glasser; Germany). The medium was changed twice a week. After three weeks the fibroblasts (hereinafter, Fibro) were separated and passaged using the 0.25% EDTA solution (Gibco; USA).

Assessment of PBMC activation and degranulation after their co-cultivation with the chondrocyte-like cells

To obtain the peripheral blood mononuclear cells (PBMCs), 9 mL of blood collected from the healthy donor were 2-fold diluted with PBS, carefully layered onto the Ficoll-Paque™ PLUS with the density of 1.077 g/cm^3 (GE Healthcare; USA), and centrifuged for 30 min at 350 g. The interphase layer was separated and washed twice with PBS. The PBMCs collected were enumerated with the Luna automated counter and diluted with the medium (X-VIVO™ 15 (Lonza; Switzerland) + 100 U/mL IL2 + 10% FBS inactivated by heating) to a concentration of 1 million cells per milliliter.

To perform analysis, each of the target cell lines (iPS, iPS_dB2M, Fibro, Chondro, iCh, and iCh_dB2M) was seeded into three wells of the 96-well plate containing appropriate culture medium. After the monolayer was formed, the medium was removed, and 200 μL of the PBMC suspension were added to the well. The plate was transferred to the incubator at +37 °C and 5% CO_2.

To assess $CD8^+$ T cell activation one day and five days later, the cells were resuspended and collected for further flow cytometry analysis.

To assess $CD8^+$ T cell degranulation, Brefeldin A with a concentration of 100 ng/mL was added to the well after 1 h of incubation, and the cells were incubated at +37 °C and 5% CO_2 for another 4 h. Then the cells were collected for flow cytometry analysis.

Flow cytometry

To assess $CD8^+$ T cell activation, the following antibodies were used: CD3-PERCP-Cy5.5 (Sony; Japan), CD8-BrilliantViolet421 (BD Bioscience; USA), CD69-FITC (Sony; Japan)

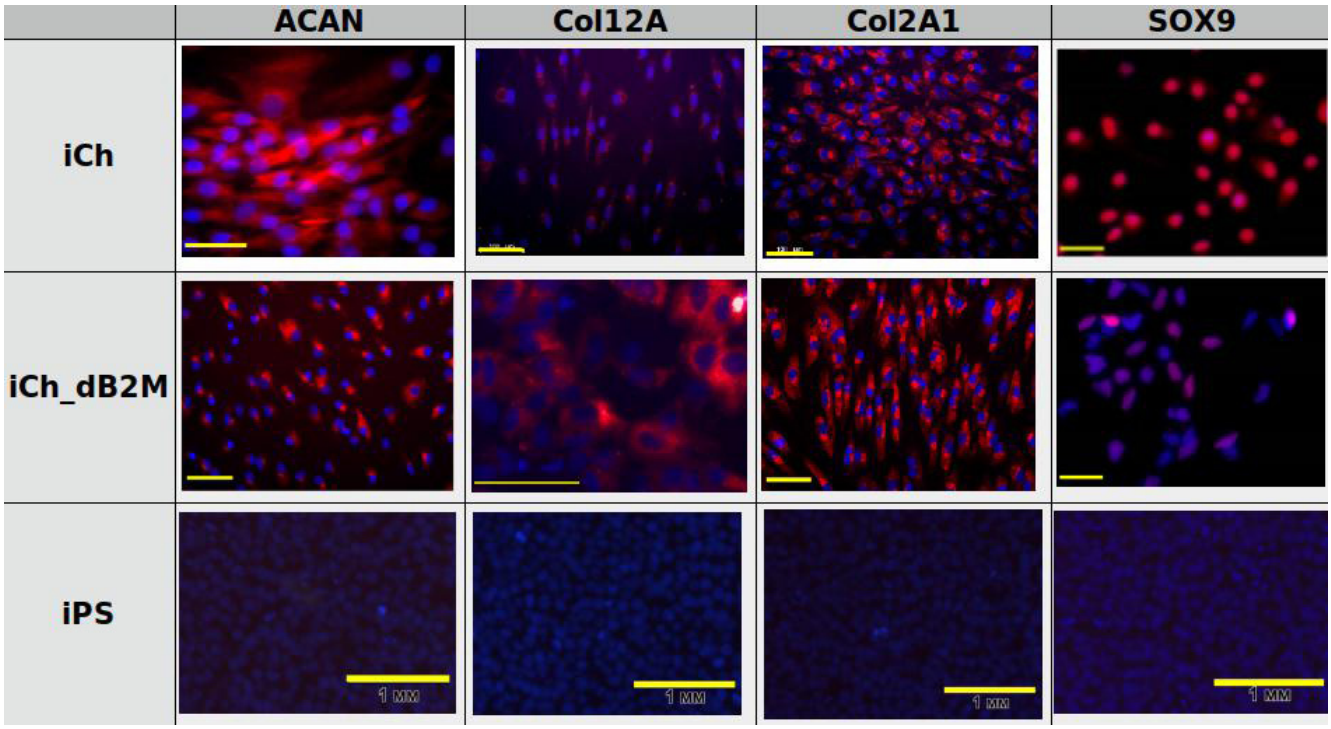

Fig. 1. Immunohistochemical assessment of monolayer cultures. The cell nuclei are stained *blue*, the studied markers are stained *red* and *green*. The scale bar size (shown in *yellow*) is 100 μm for iCh and iCh_dB2M, 1000 μm for iPS

The CD8-BrilliantViolet421 (BD Bioscience, USA), CD3-PE (Abcam, UK), CD107a-APC (Sony; Japan) antibodies were used to assess $CD8^+$ T cell degranulation. Flow cytometry analysis was performed with the NovoCyte Flow Cytometer. The cytometry data were processed using the FlowJo software tool (Tree Star Inc.; USA).

Statistical analysis

The unpaired Student's t-test and ANOVA were used to compare the fractions of activated and degranulated $CD8^+$ T cells. The differences were considered to be significant when *p*-value was below 0.05. Calculation was performed using a personal computer with the Microsoft Excel 2010 (Microsoft Corp; USA) and SPSS Statistics 17.0 (IBM; USA) software.

RESULTS

Quality control of the iPSC-derived chondrocytes

The chondrocyte-like cells obtained by directed differentiation of the B2M knockout and wild-type iPSCs were tested for expression of the major chondrogenic markers. The analysis of immunohistochemical labeling revealed fluorescence of the chondrocyte matrix proteins, type I and II collagens (COL1, COL2), aggrecan (ACAN), and the SOX9 nuclear protein in both cell lines (Fig. 1). However, iPSCs were not stained for these markers.

The real time PCR also demonstrated expression of these chondrogenic markers in both B2M knockout derivatives and wild-type ones. Furthermore, the type I collagen expression in the iCh_dB2M cells was lower than in the control group of native human chondrocytes, while the expression of type II collagen, on the contrary, was higher (Fig. 2). Furthermore, the B2M knockout iPSC derivatives showed lower ACAN and SOX9 expression compared to human chondrocytes, as well as lower fluorescence intensity compared to the cell line without knockout. No significant differences in expression of the studied chondrogenic markers from the control group were reported for the iCh cells.

Assessment of the iPSC-derived chondrocytes' immunogenicity

MHC I (major histocompatibility complex class I) is expressed in almost all cells of the body. Presentation of endogenous peptides to T cells is the main function of this protein. The genes encoding MHC I are highly polymorphic and vary between individuals, just like the set of peptides capable of presenting in appropriate MHC I molecule. During maturation the $CD8^+$ T cells acquire tolerance to their own endogenous peptides and MHC I. However, when foreign cells enter the body, the likelihood of nonspecific recognition of the peptide–MHC I complex by the T-cell receptor is high. That is why attention was paid mainly to the $CD8^+$ T cell response.

To assess the iCh-dB2M immunogenicity, this cell line was co-cultured with PBMCs of a healthy donor. After the 5-day co-cultivation we determined the percentage of activated $CD69^+$-$CD8^+$ T cells. CD69, the membrane-bound type II C-lectin receptor, is widely used as an early marker of lymphocyte activation. The CD69 expression is induced promptly on the T cell surface after binding of the T-cell receptor and CD3, which results in the cytokine secretion and proliferation of activated cells. A number of studies report the fact that CD69 expression on the T cells reaches its maximum 24 h after stimulation and then starts to decline [16, 17]. That is why we conducted another experiment, during which we measured T cell activation 24 h after the start of co-cultivation.

The flow cytometry analysis results are provided in Fig. 3. Fibroblasts of a healthy donor were used as a positive control, while iPSCs were used as a negative control due to their decreased immunogenicity [18]. Regardless of the co-cultivation duration, the percentage of $CD8^+$ T cells activated during co-cultivation with iCh-dB2M did not differ significantly from that observed during co-cultivation with

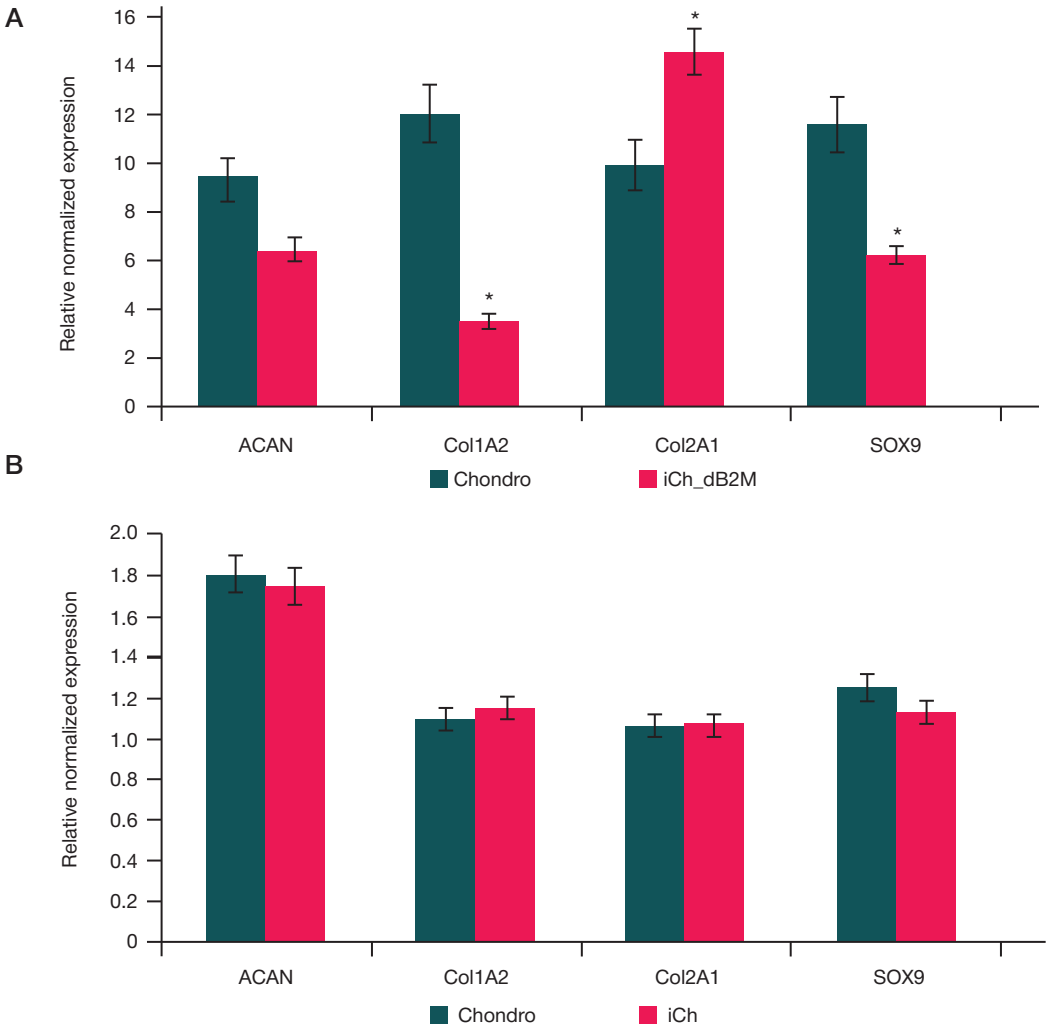

Fig. 2. Relative expression of chondrocytic markers in the human chondrocyte lines (Chondro), B2M knockout (iCh_dB2M) **(A)** and wild-type chondrocyte-like cells (iCh) **(B)**. * — significant differences in gene expression relative to human chondrocytes, $p < 0.05$, unpaired Student's *t*-test

iCh and chondrocytes, but was higher than that observed during co-cultivation with iPS or without co-cultivation. On the one hand, this may indicate that the B2M knockout is not enough for the cells to eventually become hypoimmunogenic [11, 19]. On the other hand, the 5-day co-cultivation of all the cell lines used resulted in activation of the significantly lower percentage of $CD8^+$ T cells ($p < 0.01$, unpaired *t*-test; the data are not provided) compared to fibroblasts, which is probably indicative of decreased immunogenicity of the chondrocyte-like cells and chondrocytes per se. According to the literature data, chondrocytes are capable of creating anti-inflammatory microenvironment around them, which is likely to affect the experimental results [20, 21]. At the same time, the 1-day co-cultivation with fibroblasts of a healthy donor did not result in activation of $CD8^+$ T cells. Perhaps, several factors contributed to such results. First, according to flow cytometry data, the MHC I expression on the fibroblasts of this donor was decreased compared to other fibroblast lines represented in our laboratory (the data are not provided). Second, partial match of the MHC I allele in PBMCs and fibroblasts could affect the experimental results. As a result, in case of 5-day co-cultivation, the cumulative effect resulting in activation of the significantly larger number of $CD8^+$ T cells was observed, while the 24 h stimulation was not enough for this cell line.

Thus, the iPSC-derived chondrocytes are capable of activating $CD8^+$ T cells of PBMCs. Furthermore, the B2M knockout does not have a significant effect on immunogenicity of the iPSC-derived chondrocytes.

To assess immunogenicity of the chondrocyte-like cells, the $CD8^+$ T cell cytotoxic response was also measured during co-cultivation of cell lines with PBMCs.

CD107a (LAMP-1) is a lysosomal-associated membrane glycoprotein. When the $CD8^+$ T cell is degranulated, the lysosomal granule containing the effector lytic molecules fuses with the external membrane of the $CD8^+$ T cell. As a result, the granule content is delivered to the target cell, and CD107a is on the cell surface and becomes available for staining with antibodies, as a cytotoxicity marker.

After the 5 h co-cultivation with the target cell lines and Brefeldin A, PBMCs were removed and stained with antibodies against CD3, CD8, and CD107. After that flow cytometry analysis was performed.

The data obtained are generally similar to the previous two results (Fig. 4). The CD107a expression and, therefore, cytotoxic activity of $CD8^+$ T cells observed in the presence of B2M knockout chondrocyte-like cells, chondrocytes, and fibroblasts was significantly higher ($p < 0.05$; unpaired Student's *t*-test) than that observed in the presence of iPS and no target cell line. This suggests that the cell lines we have selected are immmunogenic. It is interesting that the B2M knockout iPS also caused a cytotoxic response. We have found no similar experimental data in the literature. Given the fact that the similar trend towards an increase in immunogenicity in iPS_dB2M compared to iPS was observed throughout all three experiments, it can be assumed that the results obtained

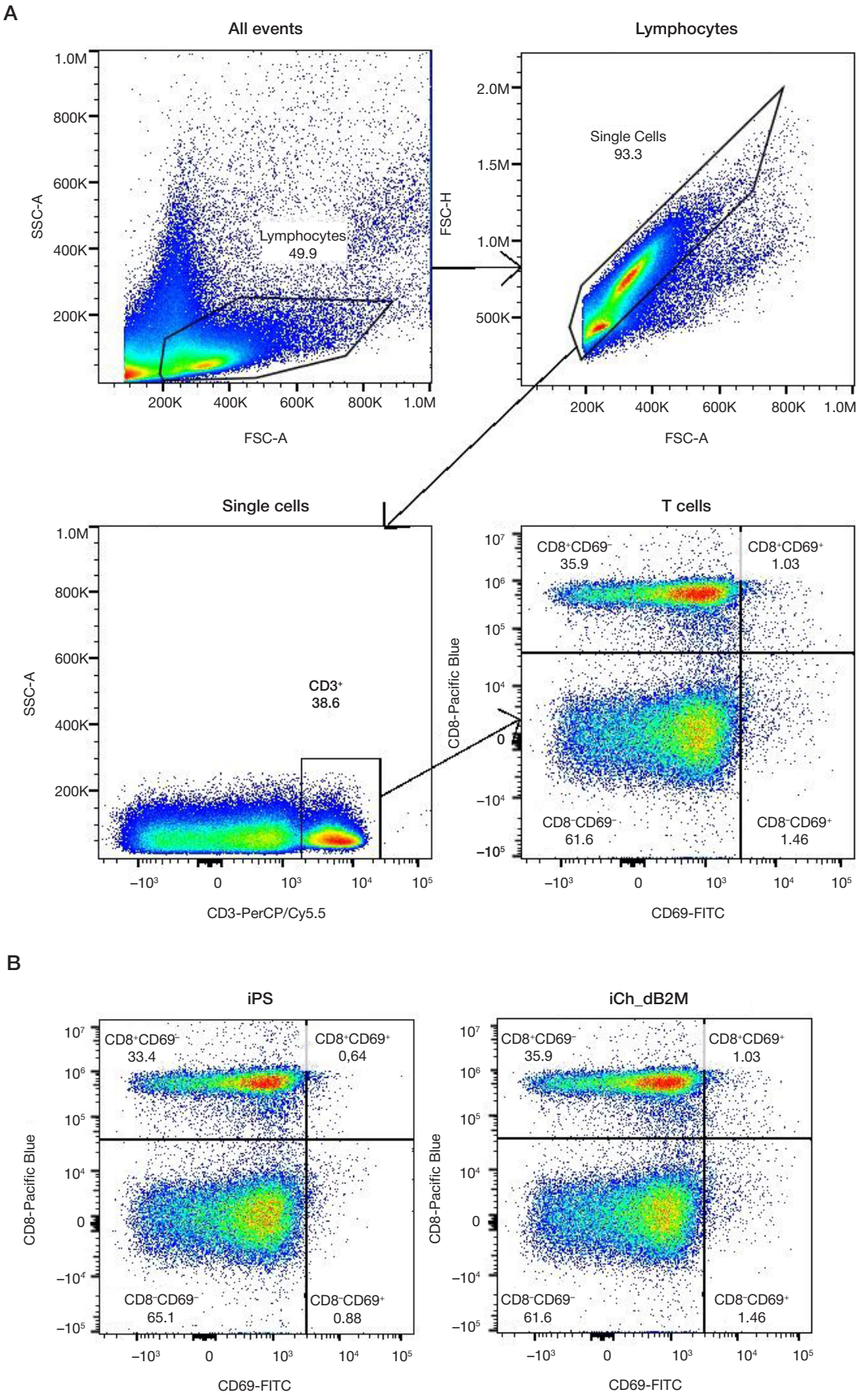

Fig. 3. Flow cytometry analysis of the CD8⁺ T cell activation. **A**. Gating scheme. **B**. Percentage of activated CD8⁺ T cells during co-cultivation with iPS (*on the left*) and iCh-dB2M (*on the right*). **C**. Comparative analysis of CD8⁺ T cell activation after the 5-day co-cultivation with the target cell lines. **D**. Comparative analysis of CD8⁺ T cell activation after the 24 h co-cultivation with the target cell lines. * — $p < 0.05$; ** — $p < 0.01$, unpaired Student's *t*-test

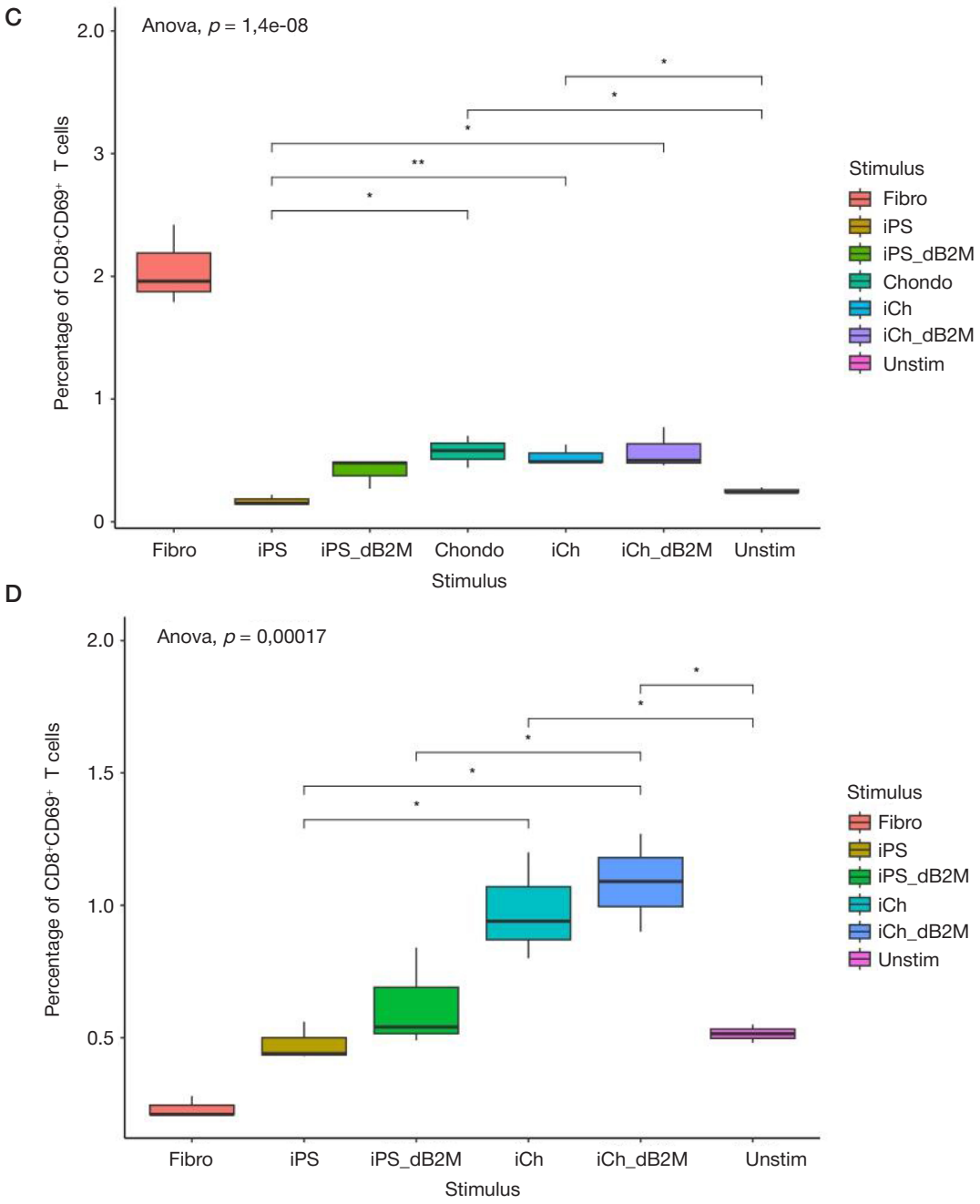

Fig. 3. (Continuation) Flow cytometry analysis of the CD8+ T cell activation. **A**. Gating scheme. **B**. Percentage of activated CD8+ T cells during co-cultivation with iPS (*on the left*) and iCh-dB2M (*on the right*). **C**. Comparative analysis of CD8+ T cell activation after the 5-day co-cultivation with the target cell lines. **D**. Comparative analysis of CD8+ T cell activation after the 24 h co-cultivation with the target cell lines. * — $p < 0.05$; ** — $p < 0.01$, unpaired Student's *t*-test

are not an artifact for this line. Then, the B2M knockout has probably somehow affected the cell line transcriptome [22]. In the future we plan to test the iPS-dB2M transcriptome and compare it to that of the original cell line.

DISCUSSION

The use of cell products for the cartilage tissue repair is currently among the most promising and efficient therapy types [23–25]. However, the limited amounts of the cartilage cell material represent a serious obstacle for the widespread use of such treatment. In recent years, it has become possible to obtain the cartilage cells via differentiation of iPSCs, which has made it significantly easier to obtain the amount of the source of autologous cells essential for creation of the graft. However, this procedure is expensive, it takes several months and requires validation. Creation of the universal hypoimmunogenic graft would help solve the problem of insufficient cell material for *in vitro* creation of the cartilage tissue. Nevertheless, biosafety of such product is poorly understood.

We have obtained the B2M knockout and wild-type iPSC-derived chondrocytes and assessed the expression of chondrogenic markers. The expression of such markers, as SOX9, aggrecan, and type I and II collagens suggests that the iPSC derivatives have acquired a chondrocytic phenotype, which was observed in both lines of derivatives during our experiment. It is worth noting that based on the real time PCR data the expression of Col1 in iCh_dB2M was lower and the expression of Col2 was higher than in human chondrocytes. This was confirmed by predominance of the hyaline cartilage phenotype among the differentiated B2M knockout derivatives, which was more preferable in terms of future clinical use [26]. At the same

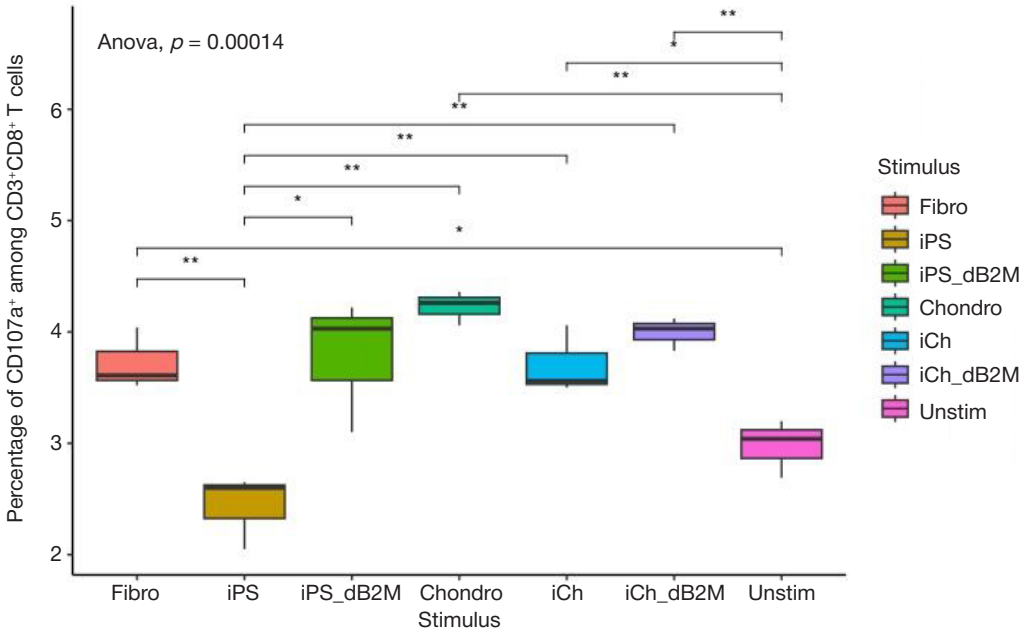

Fig. 4. Comparative analysis of CD8⁺ T cell degranulation after the 5 h co-cultivation with the target cell lines. Unpaired Student's *t*-test, * — $p < 0.05$; ** — $p < 0.01$

time, this could be indicative of acquiring fibrotic characteristics of native human chondrocytes due to cultivation [27]. Despite the fact that the Sox9 expression in iCh_B2M was lower than in iCh, we have no reason to believe that this had a significant effect on the iPSC-derived chondrocytes' functioning [28, 29].

The total removal of MHC I from the cell surface should have resulted in the chondrocyte-like cells' immunogenicity reduced due to escaping recognition of the MHC I–autopeptide complex by the CD8⁺ T cells. However, our findings have shown that knocking out B2M is not enough to reduce immunogenicity of iPSC-derived chondrocytes. In all three of our experiments, the CD8⁺ T cell immune response to the B2M-deficient chondrocyte-like cells was significantly higher than that to iPSCs. There are several mechanisms, which could underlie the CD8⁺ T cell activation.

According to one of those, CD8⁺ T cells were activated not due to direct interaction with the chondrocyte-like cells, but due to the fact that there were antigen-presenting cells (APCs) among PBMCs. As is known, the APCs are capable of presenting peptides of the absorbed particles in the context of MHC I and MHC II. To ensure activation of CD8⁺ T cells (and, therefore, expression of CD69), it is enough to recognize a foreign peptide on the APC and, in some cases, receive co-stimulation from CD4⁺ T cells [30, 31]. As for degranulation, it has been shown that CD8⁺ T cells are capable of killing the tumor cells that have lost MHC I. Such an effect was achieved in the presence of APCs, regardless of NK cells. Recognition is accomplished via binding of the NKG2D T cell receptor with the ligands (NKG2DL) on the target cells, while killing occurs due to granzyme secretion [32]. At the same time, Bogomiakova et al. have shown that the iPS fibroblast derivatives express 1.5 times more NKG2D ligands on their surface than fibroblasts of a healthy human [14]. Thus, it can be assumed that the chondrocyte-like cells used in our experiment increased expression of the NKG2D ligands, which made them a potential target for both CD8⁺ T cells and NK cells. Despite the fact that chondrocytes (and probably iPSC-derived chondrocytes) create the anti-inflammatory environment around them [20, 21], this is probably not an absolute guarantee of no immune response. It must be remembered that chondrocytes that have got into the pro-inflammatory environment (which is inevitable in case of the tissue-engineered cartilage graft transplantation) are capable of expressing MHC II [21]. Despite the fact that it has not been demonstrated *in vitro* for the iPSC-derived chondrocytes [27], no *in vivo* tests have been performed. That is why it is necessary to ensure the MHC II knockout in the iPSC-derived chondrocytes in order to avoid the CD4⁺ T cell immune response. In addition, a number of studies demonstrate successful acquisition of hypoimmunogenic iPS derivatives due to knockout of MHC I, MHC II and CD47 hyperexpression aimed at regulating the NK cell response [11, 12]. These data, in total, leave the room for the possibility of the cartilage tissue graft improvement based on the differentiated iPSC derivatives.

CONCLUSIONS

The iPSC-derived chondrocytes obtained show the same low level of immunogenicity, as human chondrocytes, however, these cause a higher immune response compared to hypoimmunogenic iPSCs. On the one hand, this suggests that the chondrocytes obtained can be a potential source of product for treatment of the articular cartilage tissue, but on the other hand this study demonstrates potential risks associated with instability of tissue after editing and avoidance of the immune response in case of tumorgenesis. Thus, the B2M knockout is not a sufficient condition for immunogenicity of the prototypes obtained from such cells of the tissue, and the iPSC-derived chondrocytes require further modification.

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