

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

Barinova AA<sup>1</sup>✉, Pikina AS<sup>1</sup>, Golubinskaya PA<sup>1</sup>, Ruchko ES<sup>2</sup>, Ereemeev AV<sup>1,2</sup>

<sup>1</sup> Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency, Moscow, Russia

<sup>2</sup> Koltzov Institute of Developmental Biology of Russian Academy of Sciences, Moscow, Russia

Today, the cell-based technologies are one of the instruments used for the cartilage tissue repair. Creation of a universal hypoinmunogenic 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<sup>+</sup> 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 hypoinmunogenic non-edited iPSCs. The B2M knockout iPSCs demonstrated a trend towards greater activation of CD8<sup>+</sup> 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

**Funding:** chondrocyte-like cells were obtained from iPSCs within the framework of the State Assignment #122032300191-2 "Organoid-22". Immunocytochemical and PCR-based assessment of chondrogenic markers in the iPSC-derived chondrocytes, as well as assessment of immunogenicity of these chondrocyte-like cells were conducted within the framework of the RSF project #22-15-00250 "Comparison of chondrogenic potential of the cartilage tissue obtained using primary chondrocyte culture and using differentiated derivatives of induced pluripotent stem cells".

**Acknowledgements:** the authors express their gratitude to M. Bogomyakova, L. Belikova, Laboratory of Cell Biology, Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia, for assistance in planning and conducting the experiment.

**Author contribution:** Barinova AA — immunological tests, manuscript preparation and writing, search for literature; Pikina AS — collecting chondrocytes from the patient and iPSC differentiation into chondrocyte-like cells, immunohistochemistry; Golubinskaya PA — search for literature; Ruchko ES — conducting PCR; Ereemeev AV — manuscript editing.

**Compliance with ethical standards:** the study was approved by the Ethics Committee of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia (protocol № 2019/02 dated 09 April 2019).

✉ **Correspondence should be addressed:** Anna A. Barinova  
Malaya Pirogovskaya, 1a, 119435, Moscow, Russia; barinova.anna.al@mail.ru

**Received:** 15.11.2023 **Accepted:** 15.01.2024 **Published online:** 05.02.2024

**DOI:** 10.47183/mes.2024.001

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

А. А. Баринаова<sup>1</sup>✉, А. С. Пикина<sup>1</sup>, П. А. Голубинская<sup>1</sup>, Е. С. Ручко<sup>2</sup>, А. В. Еремеев<sup>1,2</sup>

<sup>1</sup> Федеральный научно-клинический центр физико-химической медицины имени Ю. М. Лопухина Федерального медико-биологического агентства, Москва, Россия

<sup>2</sup> Институт биологии развития имени Н. К. Кольцова, Москва, Россия

В настоящее время клеточные технологии являются одним из инструментов по восстановлению хрящевой ткани. Создание универсального гипоиimmunогенного 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<sup>+</sup>-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 гипоиimmunогенных нередактированных ИПСК. 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 производных 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».

**Благодарности:** М. Богомяковой, Л. Беликовой из лаборатории клеточной биологии ФНКЦ ФХМ имени Ю. М. Лопухина ФМБА России за помощь в планировании и проведении эксперимента.

**Вклад авторов:** А. А. Баринаова — проведение иммунологических тестов, подготовка и написание статьи, поиск литературы; А. С. Пикина — получение хондроцитов пациента и дифференцировка ИПСК в хондроцитарные производные, проведение ИЦХ; П. А. Голубинская — поиск литературы; Е. С. Ручко — проведение ПЦР; А. В. Еремеев — редактирование статьи.

**Соблюдение этических стандартов:** исследование одобрено этическим комитетом ФНКЦ ФХМ (протокол № 2019/02 от 09 апреля 2019 г.).

✉ **Для корреспонденции:** Анна Александровна Баринаова  
ул. Малая Пироговская, д. 1а, 119435, г. Москва, Россия; barinova.anna.al@mail.ru

**Статья получена:** 15.11.2023 **Статья принята к печати:** 15.01.2024 **Опубликована онлайн:** 05.02.2024

**DOI:** 10.47183/mes.2024.001

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<sup>+</sup> T cells [10].

Creation and the use of cartilage grafts based on allogeneic 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 hypoinmunogenic grafts based on the MHC knockout iPSCs [11, 12]. It has been

shown that the allogeneic 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<sub>2</sub> incubators at +37 °C and 5% CO<sub>2</sub> until a monolayer was formed. All further incubations were also carried out in the CO<sub>2</sub> incubators at +37 °C and 5% CO<sub>2</sub>. 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<br>R: CACGTCGCGGAAGTCGATAG    | 283              |
| ACAN   | F: AGGAGTCCCTGACCTGGTTT<br>R: CCTGACAGATCTGCCTCTCC    | 167              |
| COL1A2 | F: AGGGTGAGACAGGCGAACA<br>R: CCGTTGAGTCCATCTTTGC      | 184              |
| COL2A1 | F: TGGACGCCATGAAGGTTTCT<br>R: CCATTGATGGTTTCTCAAAC    | 142              |
| YWHAZ  | F: ACTTTTGGTACATTGTGGCTTCAA<br>R: CCGCCAGGACAAACAGTAT | 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 Olympus 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  $\Delta\Delta C_t$  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<sub>2</sub> 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<sub>2</sub> 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 placed 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<sup>3</sup> (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.

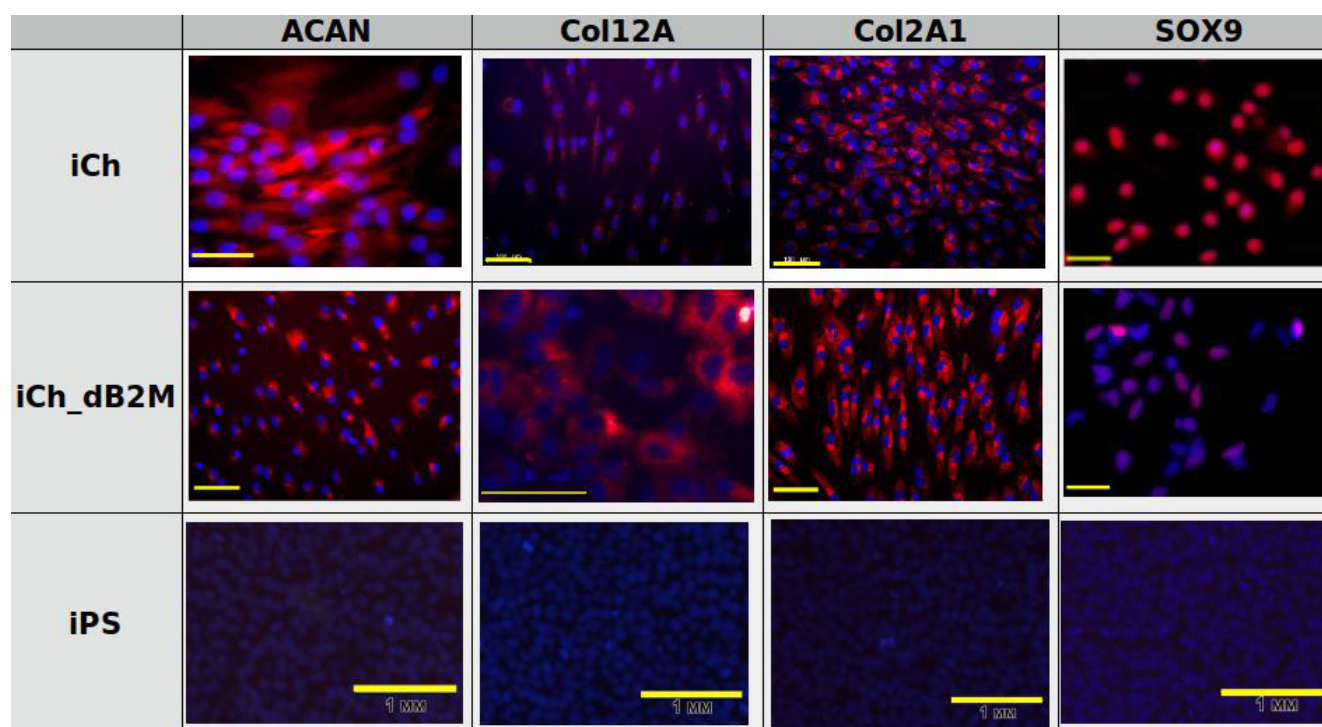
To perform analysis, each of the target cell lines (iPS, iPS\_dB2M, Fibro, Chondro, iCh, and iCh\_dB2M) was seeded into three wells of the 96-well plate containing appropriate culture medium. After the monolayer was formed, the medium was removed, and 200 µL of the PBMC suspension were added to the well. The plate was transferred to the incubator at +37 °C and 5% CO<sub>2</sub>.

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

To assess CD8<sup>+</sup> 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<sub>2</sub> for another 4 h. Then the cells were collected for flow cytometry analysis.

### Flow cytometry

To assess CD8<sup>+</sup> 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  $\mu$ m for iCh and iCh\_dB2M, 1000  $\mu$ m for iPS

The CD8-BrilliantViolet421 (BD Bioscience, USA), CD3-PE (Abcam, UK), CD107a-APC (Sony; Japan) antibodies were used to assess CD8<sup>+</sup> 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<sup>+</sup> 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

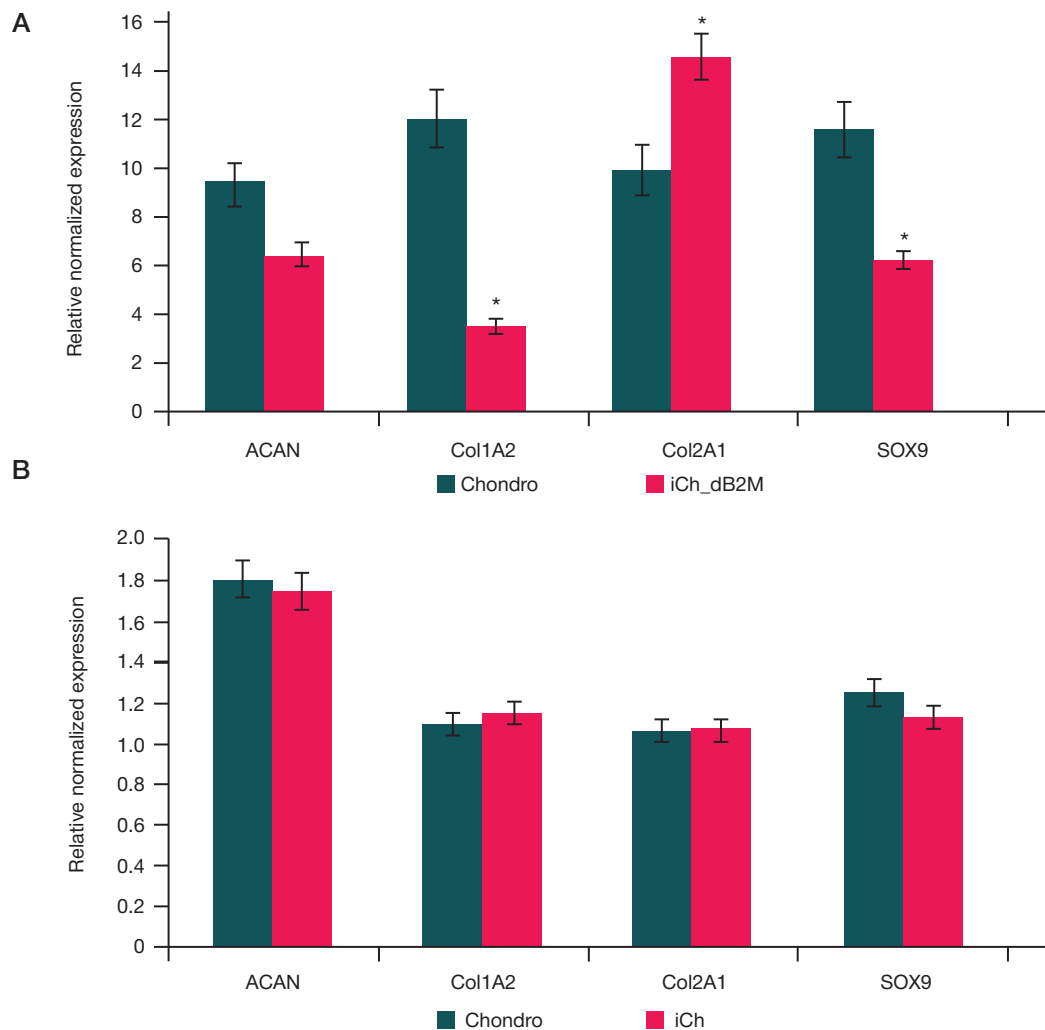
the studied chondrogenic markers from the control group were reported for the iCh cells.

### 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>-CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells was observed, while the 24 h stimulation was not enough for this cell line.

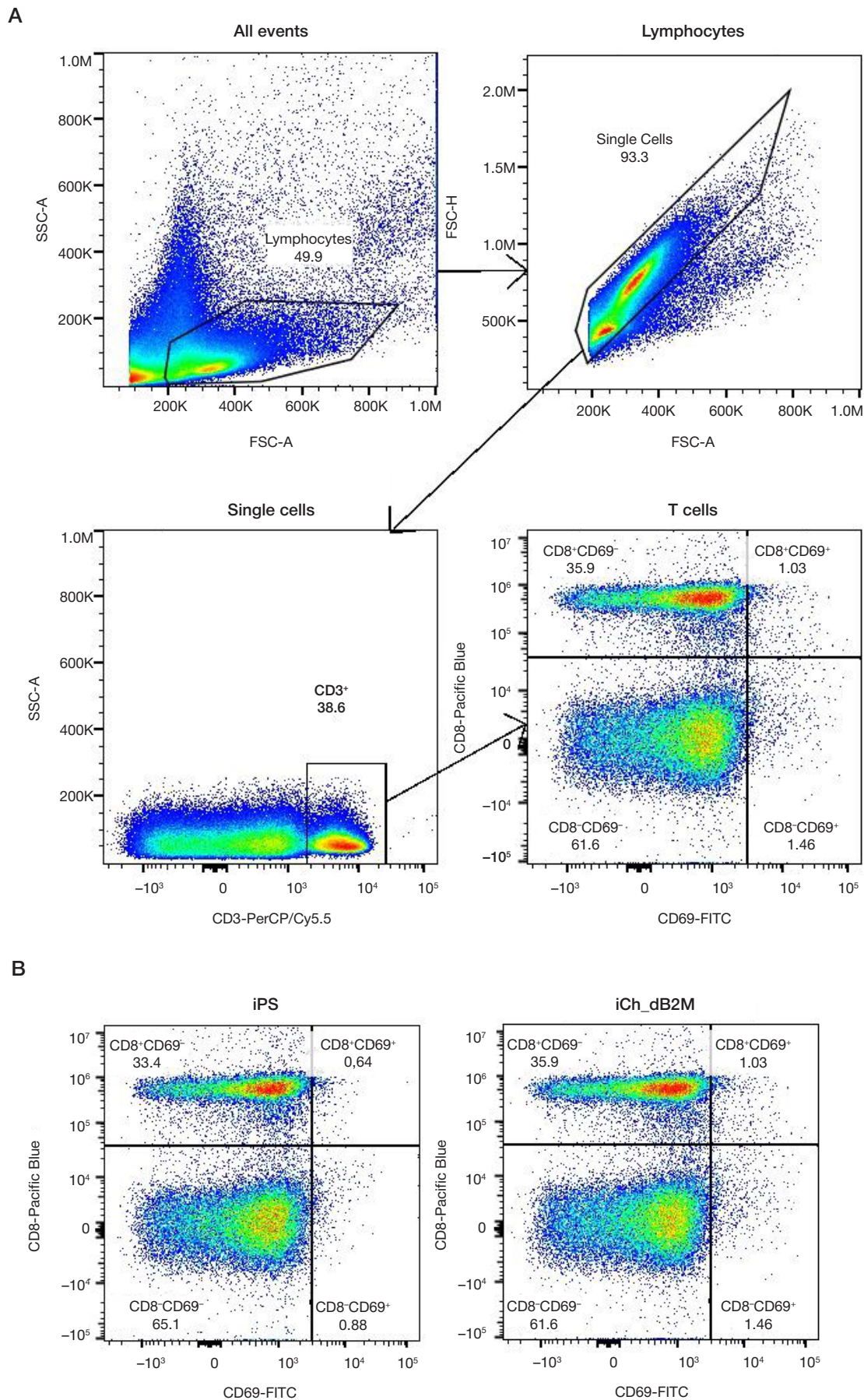
Thus, the iPSC-derived chondrocytes are capable of activating CD8<sup>+</sup> T cells of PBMCs. Furthermore, the B2M knockout does not have a significant effect on immunogenicity of the iPSC-derived chondrocytes.

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

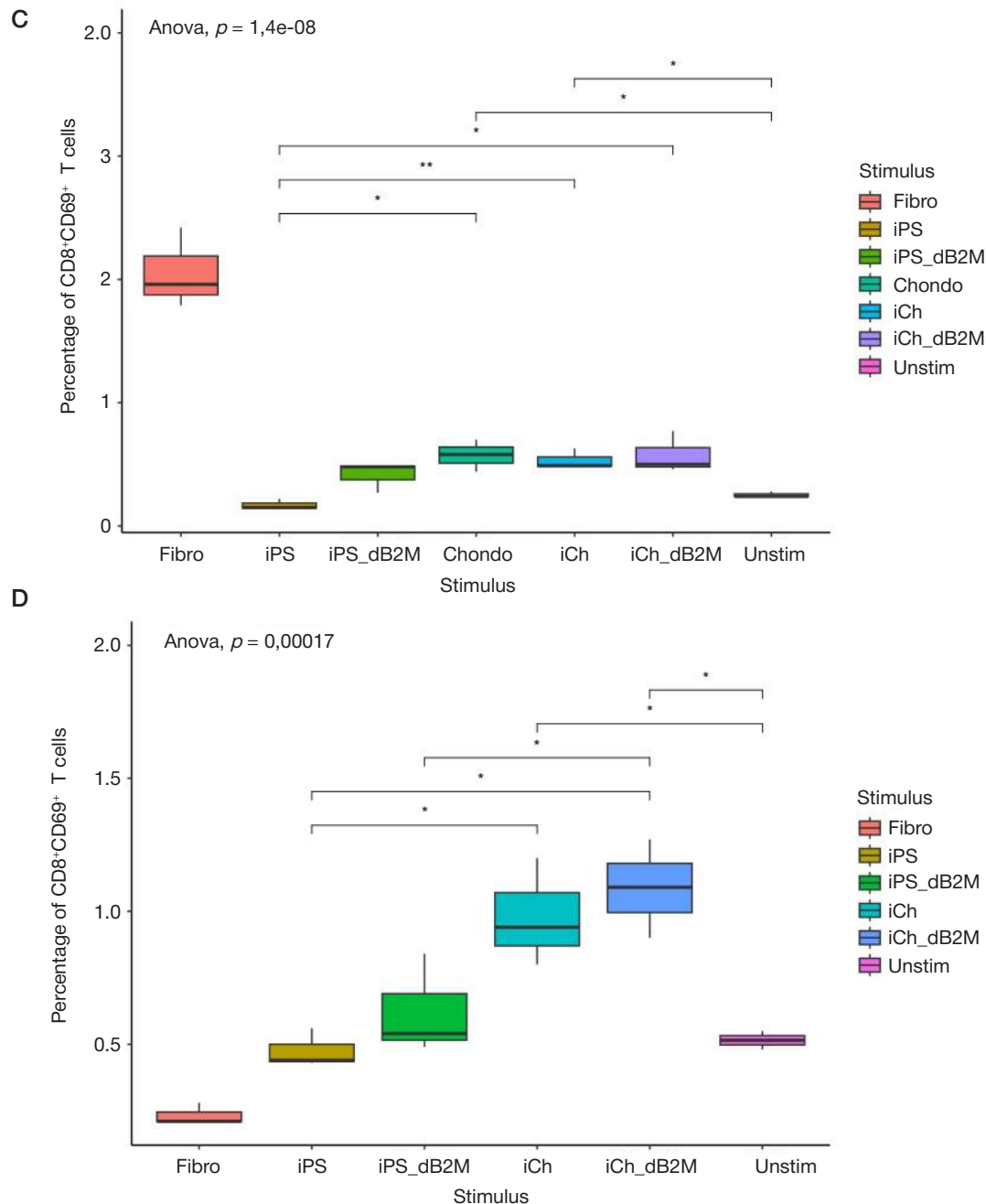
CD107a (LAMP-1) is a lysosomal-associated membrane glycoprotein. When the CD8<sup>+</sup> T cell is degranulated, the lysosomal granule containing the effector lytic molecules fuses with the external membrane of the CD8<sup>+</sup> T cell. As a result, the granule content is delivered to the target cell, 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<sup>+</sup> 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 immunogenic. 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<sup>+</sup> T cell activation. **A.** Gating scheme. **B.** Percentage of activated CD8<sup>+</sup> T cells during co-cultivation with iPS (on the left) and iCh-dB2M (on the right). **C.** Comparative analysis of CD8<sup>+</sup> T cell activation after the 5-day co-cultivation with the target cell lines. **D.** Comparative analysis of CD8<sup>+</sup> 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<sup>+</sup> T cell activation. **A.** Gating scheme. **B.** Percentage of activated CD8<sup>+</sup> T cells during co-cultivation with iPS (on the left) and iCh-dB2M (on the right). **C.** Comparative analysis of CD8<sup>+</sup> T cell activation after the 5-day co-cultivation with the target cell lines. **D.** Comparative analysis of CD8<sup>+</sup> 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

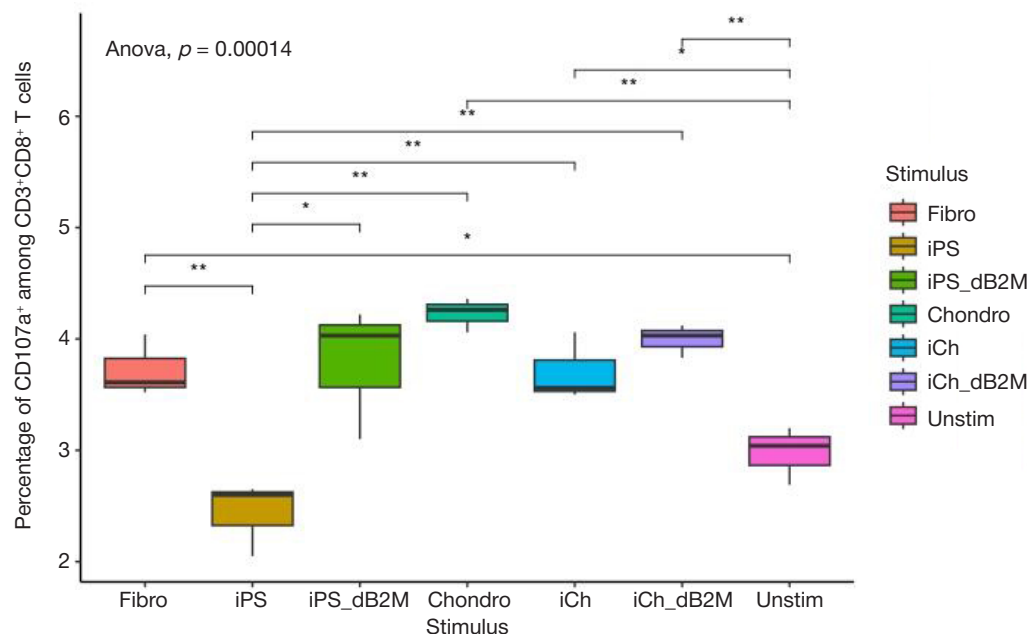
are not an artifact for this line. Then, the B2M knockout has probably somehow affected the cell line transcriptome [22]. In the future we plan to test the iPS-dB2M transcriptome and compare it to that of the original cell line.

## 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<sup>+</sup> 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\_dB2M 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell activation.

According to one of those, CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells [30, 31]. As for degranulation, it has been shown that CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell immune response. In addition, a number of studies demonstrate successful acquisition of hypoimmunogenic iPSC 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 tumorigenesis. 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.

## References

- Kabalyk MA. Rasprostranennost' osteoartrita v Rossii: regional'nye aspekty dinamiki statisticheskikh pokazatelej za 2011–2016 gg. Nauchno-prakticheskaja revmatologija. 2018; 56 (4): 416–22. Dostupno po ssylke: <https://doi.org/10.14412/1995-4484-2018-416-422>. Russian.
- Medvedeva EV, Grebenik EA, Gornostaeva SN, et al. Repair of damaged articular cartilage: current approaches and future directions. *Int J Mol Sci*. 2018; 19 (8). DOI: 10.3390/ijms190823660
- Pintan GF, de Oliveira ASJ, Lenza M, Antonioli E, Ferretti M. Update on biological therapies for knee injuries: osteoarthritis. *Curr Rev Musculoskelet Med*. 2014; 7 (3): 263–9. DOI: 10.1007/s12178-014-9229-8.
- Martinčić D, Leban J, Filardo G, et al. Autologous chondrocytes versus filtered bone marrow mesenchymal stem/stromal cells for knee cartilage repair—a prospective study. *Int Orthop*. 2021; 45 (4): 931–9. DOI: 10.1007/s00264-020-04727-2.
- Leigheb M, Bosetti M, De Consoli A, Borroni A, Cannas M, Grassi F. Chondral tissue engineering of the rheumatoid knee with collagen matrix autologous chondrocytes implant. *Acta Biomed*. 2017; 88 (4S): 107–13. DOI: 10.23750/abm.v88i4-S.6801.
- Davies RL, Kuiper NJ. Regenerative medicine: a review of the evolution of autologous chondrocyte implantation (ACI) therapy. *Bioeng (Basel, Switzerland)*. 2019; 6 (1). DOI: 10.3390/bioengineering6010022.
- Vonk LA, de Windt TS, Kragten AHM, et al. Enhanced cell-induced articular cartilage regeneration by chondrons; the influence of joint damage and harvest site. *Osteoarthr Cartil*. 2014; 22 (11): 1910–7. DOI: 10.1016/j.joca.2014.08.005.
- Khan NM, Diaz-Hernandez ME, Chihab S, et al. Differential chondrogenic differentiation between iPSC derived from healthy and OA cartilage is associated with changes in epigenetic regulation and metabolic transcriptomic signatures. *Elife*. 2023; 12. DOI: 10.7554/eLife.83138.
- Viñuelas R, Sanjurjo-Rodríguez C, Piñeiro-Ramil M, et al. Generation and characterization of human induced pluripotent stem cells (iPSCs) from hand osteoarthritis patient-derived fibroblasts. *Sci Rep*. 2020; 10. DOI: 10.1038/s41598-020-61071-6.
- Abe K, Yamashita A, Morioka M, et al. Engraftment of allogeneic iPS cell-derived cartilage organoid in a primate model of articular cartilage defect. *Nat Commun*. 2023; 14 (1): 804. DOI: 10.1038/s41467-023-36408-0.
- Deuse T, Hu X, Gravina A, et al. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nat Biotechnol*. 2019; 37 (3): 252–8. DOI: 10.1038/s41587-019-0016-3.
- Trionfini P, Romano E, Varinelli M, et al. Hypoimmunogenic human pluripotent stem cells as a powerful tool for liver regenerative medicine. *Int J Mol Sci*. 2023; 24 (14). DOI: 10.3390/ijms241411810.
- Okutani Y, Abe K, Yamashita A, Morioka M, Matsuda S, Tsumaki N. Generation of monkey induced pluripotent stem cell-derived cartilage lacking major histocompatibility complex class I molecules on the cell surface. *Tissue Eng Part A*. 2022; 28 (1–2): 94–106. DOI: 10.1089/ten.TEA.2021.0053.
- Bogomiakova ME, Sekretova EK, Anufrieva KS, et al. iPSC-derived cells lack immune tolerance to autologous NK-cells due to imbalance in ligands for activating and inhibitory NK-cell receptors. *Stem Cell Res Ther*. 2023; 14 (1): 77. DOI: 10.1186/s13287-023-03308-5.
- Bogomiakova ME, Sekretova EK, Ereemeev AV, et al. Derivation of induced pluripotent stem cells line (RCPCMi007-A-1) with inactivation of the beta-2-microglobulin gene by CRISPR/Cas9 genome editing. *Stem Cell Res*. 2021; 55: 102451. Available from: <https://doi.org/10.1016/j.scr.2021.102451>.
- Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin Diagn Lab Immunol*. 1996; 3 (3): 301–4. DOI: 10.1128/cdli.3.3.301-304.1996.
- Zimmerman M, Yang D, Hu X, et al. IFN- $\gamma$  Upregulates survivin and Ifi202 expression to induce survival and proliferation of tumor-specific T cells. *PLoS One*. 2010; 5 (11): e14076. Available from: <https://doi.org/10.1371/journal.pone.0014076>.
- Yamasaki S, Sugita S, Horiuchi M, et al. Low Immunogenicity and immunosuppressive properties of human ESC- and iPSC-derived retinas. *Stem Cell Reports*. 2021; 16 (4): 851–67. Available from: <https://doi.org/10.1016/j.stemcr.2021.02.021>.
- Petrus-Reurer S, Winblad N, Kumar P, et al. Generation of retinal pigment epithelial cells derived from human embryonic stem cells lacking human leukocyte antigen class I and II. *Stem cell reports*. 2020; 14 (4): 648–62. DOI: 10.1016/j.stemcr.2020.02.006.
- Pereira RC, Martinelli D, Cancedda R, Gentili C, Poggi A. Human articular chondrocytes regulate immune response by affecting directly T cell proliferation and indirectly inhibiting monocyte differentiation to professional antigen-presenting cells. *Front Immunol*. 2016; 7. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2016.00415>.
- Osiecka-Iwan A, Hyc A, Radomska-Lesniewska DM, Rymarczyk A, Skopinski P. Antigenic and immunogenic properties of chondrocytes. Implications for chondrocyte therapeutic transplantation and pathogenesis of inflammatory and degenerative joint diseases. *Cent J Immunol*. 2018; 43 (2): 209–19. DOI: 10.5114/cej.2018.77392.
- Tsai HH, Kao HJ, Kuo MW, et al. Whole genomic analysis reveals atypical non-homologous off-target large structural variants induced by CRISPR-Cas9-mediated genome editing. *Nat Commun*. 2023; 14 (1): 5183. DOI: 10.1038/s41467-023-40901-x.
- Ramezankhani R, Torabi S, Minaei N, et al. Two decades of global progress in authorized advanced therapy medicinal products: an emerging revolution in therapeutic strategies. *Front Cell Dev Biol*. 2020; 8: 547653. DOI: 10.3389/fcell.2020.547653.
- Kim J, Park J, Song SY, Kim E. Advanced therapy medicinal products for autologous chondrocytes and comparison of regulatory systems in target countries. *Regen Ther*. 2022; 20: 126–37. DOI: 10.1016/j.reth.2022.04.004.
- Colombini A, Libonati F, Lopa S, Peretti GM, Moretti M, de Girolamo L. Autologous chondrocyte implantation provides good long-term clinical results in the treatment of knee osteoarthritis: a systematic review. *Knee Surg Sports Traumatol Arthrosc*. 2023; 31 (6): 2338–48. DOI: 10.1007/s00167-022-07030-2.
- Tsumaki N, Okada M, Yamashita A. iPS cell technologies and cartilage regeneration. *Bone*. 2015; 70: 48–54. DOI: 10.1016/j.bone.2014.07.011.
- Kimura T, Yamashita A, Ozono K, Tsumaki N. Limited Immunogenicity of Human Induced Pluripotent Stem Cell-Derived Cartilages. *Tissue Eng Part A*. 2016; 22 (23–24): 1367–75. DOI: 10.1089/ten.tea.2016.0189.
- Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Pöschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. *Matrix Biol*. 2003; 22 (4): 363–72. DOI: 10.1016/s0945-053x(03)00049-0.
- Lee J, Smeriglio P, Chu CR, Bhutani N. Human iPSC-derived chondrocytes mimic juvenile chondrocyte function for the dual advantage of increased proliferation and resistance to IL1 $\beta$ . *Stem Cell Res Ther*. 2017; 8 (1): 244. DOI: 10.1186/s13287-017-0696-x.
- Oladipo OO, Adediji BO, Adedokun SP, Gbadamosi JA, Salaudeen M. Regulation of effector and memory CD8+ T cell differentiation: a focus on orphan nuclear receptor NR4A family, transcription factor, and metabolism. *Immunol Res*. 2023; 71 (3): 314–27. DOI: 10.1007/s12026-022-09353-1.
- Henrickson SE, von Andrian UH. Single-cell dynamics of T-cell priming. *Curr Opin Immunol*. 2007; 19 (3): 249–58. DOI: 10.1016/j.coi.2007.04.013.
- Lerner EC, Woroniecka KI, D'Anniballe VM, et al. CD8+ T cells maintain killing of MHC-I-negative tumor cells through the NKG2D–NKG2DL axis. *Nat Cancer*. 2023; 4 (9): 1258–72. DOI: 10.1038/s43018-023-00600-4.

## Литература

1. Кабалык М. А. Распространенность остеоартрита в России: региональные аспекты динамики статистических показателей за 2011–2016 гг. Научно-практическая ревматология. 2018; 56 (4): 416–22. Доступно по ссылке: <https://doi.org/10.14412/1995-4484-2018-416-422>.
2. Medvedeva EV, Grebenik EA, Gornostaeva SN, et al. Repair of damaged articular cartilage: current approaches and future directions. *Int J Mol Sci.* 2018; 19 (8). DOI: 10.3390/ijms19082366.
3. Pintan GF, de Oliveira ASJ, Lenza M, Antonioli E, Ferretti M. Update on biological therapies for knee injuries: osteoarthritis. *Curr Rev Musculoskelet Med.* 2014; 7 (3): 263–9. DOI: 10.1007/s12178-014-9229-8.
4. Martinčič D, Leban J, Filardo G, et al. Autologous chondrocytes versus filtered bone marrow mesenchymal stem/stromal cells for knee cartilage repair-a prospective study. *Int Orthop.* 2021; 45 (4): 931–9. DOI: 10.1007/s00264-020-04727-2.
5. Leighab M, Bosetti M, De Consoli A, Borroni A, Cannas M, Grassi F. Chondral tissue engineering of the reumatoid knee with collagen matrix autologous chondrocytes implant. *Acta Biomed.* 2017; 88 (4S): 107–13. DOI: 10.23750/abm.v88i4-S.6801.
6. Davies RL, Kuiper NJ. Regenerative medicine: a review of the evolution of autologous chondrocyte implantation (ACI) therapy. *Bioeng (Basel, Switzerland).* 2019; 6 (1). DOI: 10.3390/bioengineering6010022.
7. Vonk LA, de Windt TS, Kragten AHM, et al. Enhanced cell-induced articular cartilage regeneration by chondrons; the influence of joint damage and harvest site. *Osteoarthr Cartil.* 2014; 22 (11): 1910–7. DOI: 10.1016/j.joca.2014.08.005.
8. Khan NM, Diaz-Hernandez ME, Chihab S, et al. Differential chondrogenic differentiation between iPSC derived from healthy and OA cartilage is associated with changes in epigenetic regulation and metabolic transcriptomic signatures. *Elife.* 2023; 12. DOI: 10.7554/eLife.83138.
9. Viñuelas R, Sanjurjo-Rodríguez C, Piñeiro-Ramil M, et al. Generation and characterization of human induced pluripotent stem cells (iPSCs) from hand osteoarthritis patient-derived fibroblasts. *Sci Rep.* 2020; 10. DOI: 10.1038/s41598-020-61071-6.
10. Abe K, Yamashita A, Morioka M, et al. Engraftment of allogeneic iPSC cell-derived cartilage organoid in a primate model of articular cartilage defect. *Nat Commun.* 2023; 14 (1): 804. DOI: 10.1038/s41467-023-36408-0.
11. Deuse T, Hu X, Gravina A, et al. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nat Biotechnol.* 2019; 37 (3): 252–8. DOI: 10.1038/s41587-019-0016-3.
12. Trionfini P, Romano E, Varinelli M, et al. Hypoimmunogenic human pluripotent stem cells as a powerful tool for liver regenerative medicine. *Int J Mol Sci.* 2023; 24 (14). DOI: 10.3390/ijms241411810.
13. Okutani Y, Abe K, Yamashita A, Morioka M, Matsuda S, Tsumaki N. Generation of monkey induced pluripotent stem cell-derived cartilage lacking major histocompatibility complex class I molecules on the cell surface. *Tissue Eng Part A.* 2022; 28 (1–2): 94–106. DOI: 10.1089/ten.TEA.2021.0053.
14. Bogomiakova ME, Sekretova EK, Anufrieva KS, et al. iPSC-derived cells lack immune tolerance to autologous NK-cells due to imbalance in ligands for activating and inhibitory NK-cell receptors. *Stem Cell Res Ther.* 2023; 14 (1): 77. DOI: 10.1186/s13287-023-03308-5.
15. Bogomiakova ME, Sekretova EK, Ereemeev AV, et al. Derivation of induced pluripotent stem cells line (RCPCM007-A-1) with inactivation of the beta-2-microglobulin gene by CRISPR/Cas9 genome editing. *Stem Cell Res.* 2021; 55: 102451. Available from: <https://doi.org/10.1016/j.scr.2021.102451>.
16. Simms PE, Ellis TM. Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clin Diagn Lab Immunol.* 1996; 3 (3): 301–4. DOI: 10.1128/cdli.3.3.301-304.1996.
17. Zimmerman M, Yang D, Hu X, et al. IFN- $\gamma$  Upregulates survivin and lfi202 expression to induce survival and proliferation of tumor-specific T cells. *PLoS One.* 2010; 5 (11): e14076. Available from: <https://doi.org/10.1371/journal.pone.0014076>.
18. Yamasaki S, Sugita S, Horiuchi M, et al. Low Immunogenicity and immunosuppressive properties of human ESC- and iPSC-derived retinas. *Stem Cell Reports.* 2021; 16 (4): 851–67. Available from: <https://doi.org/10.1016/j.stemcr.2021.02.021>.
19. Petrus-Reurer S, Winblad N, Kumar P, et al. Generation of retinal pigment epithelial cells derived from human embryonic stem cells lacking human leukocyte antigen class I and II. *Stem cell reports.* 2020; 14 (4): 648–62. DOI: 10.1016/j.stemcr.2020.02.006.
20. Pereira RC, Martinelli D, Cancedda R, Gentili C, Poggi A. Human articular chondrocytes regulate immune response by affecting directly T cell proliferation and indirectly inhibiting monocyte differentiation to professional antigen-presenting cells. *Front Immunol.* 2016; 7. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2016.00415>.
21. Osiecka-Iwan A, Hyc A, Radomska-Lesniewska DM, Rymarczyk A, Skopinski P. Antigenic and immunogenic properties of chondrocytes. Implications for chondrocyte therapeutic transplantation and pathogenesis of inflammatory and degenerative joint diseases. *Cent J Immunol.* 2018; 43 (2): 209–19. DOI: 10.5114/ceji.2018.77392.
22. Tsai HH, Kao HJ, Kuo MW, et al. Whole genomic analysis reveals atypical non-homologous off-target large structural variants induced by CRISPR-Cas9-mediated genome editing. *Nat Commun.* 2023; 14 (1): 5183. DOI: 10.1038/s41467-023-40901-x.
23. Ramezankhani R, Torabi S, Minaei N, et al. Two decades of global progress in authorized advanced therapy medicinal products: an emerging revolution in therapeutic strategies. *Front cell Dev Biol.* 2020; 8: 547653. DOI: 10.3389/fcell.2020.547653.
24. Kim J, Park J, Song SY, Kim E. Advanced therapy medicinal products for autologous chondrocytes and comparison of regulatory systems in target countries. *Regen Ther.* 2022; 20: 126–37. DOI: 10.1016/j.reth.2022.04.004.
25. Colombini A, Libonati F, Lopa S, Peretti GM, Moretti M, de Girolamo L. Autologous chondrocyte implantation provides good long-term clinical results in the treatment of knee osteoarthritis: a systematic review. *Knee Surg Sports Traumatol Arthrosc.* 2023; 31 (6): 2338–48. DOI: 10.1007/s00167-022-07030-2.
26. Tsumaki N, Okada M, Yamashita A. iPS cell technologies and cartilage regeneration. *Bone.* 2015; 70: 48–54. DOI: 10.1016/j.bone.2014.07.011.
27. Kimura T, Yamashita A, Ozono K, Tsumaki N. Limited Immunogenicity of Human Induced Pluripotent Stem Cell-Derived Cartilages. *Tissue Eng Part A.* 2016; 22 (23–24): 1367–75. DOI: 10.1089/ten.tea.2016.0189.
28. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Pöschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. *Matrix Biol.* 2003; 22 (4): 363–72. DOI: 10.1016/s0945-053x(03)00049-0.
29. Lee J, Smeriglio P, Chu CR, Bhutani N. Human iPSC-derived chondrocytes mimic juvenile chondrocyte function for the dual advantage of increased proliferation and resistance to IL1 $\beta$ . *Stem Cell Res Ther.* 2017; 8 (1): 244. DOI: 10.1186/s13287-017-0696-x.
30. Oladipo OO, Adedeji BO, Adedokun SP, Gbadamosi JA, Salaudeen M. Regulation of effector and memory CD8+T cell differentiation: a focus on orphan nuclear receptor NR4A family, transcription factor, and metabolism. *Immunol Res.* 2023; 71 (3): 314–27. DOI: 10.1007/s12026-022-09353-1.
31. Henrickson SE, von Andrian UH. Single-cell dynamics of T-cell priming. *Curr Opin Immunol.* 2007; 19 (3): 249–58. DOI: 10.1016/j.coi.2007.04.013.
32. Lerner EC, Woroniecka KI, D'Annibale VM, et al. CD8+ T cells maintain killing of MHC- I-negative tumor cells through the NKG2D–NKG2DL axis. *Nat Cancer.* 2023; 4 (9): 1258–72. DOI: 10.1038/s43018-023-00600-4.