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ACTIVATION OF HUMAN PLURIPOTENT STEM CELL-DERIVED DENDRITIC CELLS BY CANCER CELL CO-CULTURE

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КО-КУЛЬТИВИРОВАНИЕ С РАКОВЫМИ КЛЕТКАМИ АКТИВИРУЕТ ДЕНДРИТНЫЕ КЛЕТКИ, ПОЛУЧЕННЫЕ ИЗ ПЛЮРИПОТЕНТНЫХ СТВОЛОВЫХ КЛЕТОК ЧЕЛОВЕКА

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Human pluripotent stem cells (hPSCs) are considered a source of cell therapy. Dendritic cells (DCs) derived from hPSCs (hPSC-DCs) are viewed as similar to fetal DCs. The latter, while mediating immune suppression, maintain fetal homeostasis. This study looks at the outcomes of a protocol designed for massive and long-term generation of hPSC-DCs in a serum-free cell culture. When cultured in serum-free media, the newly generated pure populations of immature hPSC-DCs featured significant stability. Such DCs exhibited the typical morphology, active phagocytosis, and were highly motile. The cells expressed on the outer cell membrane specific well-known DC markers, CD11c, CD86, and CD45, while were negative for CD14, the key macrophage marker. Attempts to activate the hPSC-DCs through proinflammatory factors failed to induce IL-12 secretion and cell surface expression of HLA-DR. Yet, they elicited the expression of several genes encoding markers that belong to the conventional DC subset 1 (cDC1). Co-culturing hPSC-DCs with thyroid cancer cells entailed secretion of IL-12 and, in a fraction of the population, strong cell surface expression of HLA-DR and other markers of activated DCs. Our observations suggest that hPSC-DCs require necrotic cancer cell-derived damage-associated molecular patterns (DAMPs) for their activation.

Keywords: dendritic cells, pluripotent stem cells, hematopoiesis, differentiation, cancer cells

Плюрипотентные стволовые клетки человека (чПСК) рассматриваются в качестве источника для клеточной терапии. Дендритные клетки (ДК), полученные из чПСК (чПСК-ДК), считаются аналогами фетальных ДК. Последние, опосредуя процессы иммуносупрессии, поддерживают фетальный гомеостаз. В исследовании приведены результаты разработанного протокола массовой и длительной генерации чПСК-ДК в условиях бессывороточной клеточной культуры. Полученные чистые популяции незрелых чПСК-ДК отличались значительной стабильностью при культивации в бессывороточной среде, обладали типичной морфологией, демонстрировали заметную подвижность и способность к активному фагоцитозу. Клетки экспрессировали на внешней клеточной мембране некоторые известные маркеры ДК – CD11c, CD86, CD45 и были негативны в отношении ключевого макрофагального маркера CD14. Попытки активации чПСК-ДК с помощью провоспалительных факторов не индуцировали секрецию IL-12 и появление HLA-DR на клеточной поверхности, но приводили к экспрессии генов, кодирующих маркеры подтипа 1 конвенциональных ДК (кДК1). Однако ко-культивирование чПСК-ДК с клетками рака щитовидной железы вызывало секрецию IL-12 и – в части популяции – усиленную поверхностную экспрессию HLA-DR и других маркеров активированных ДК. Можно предположить, что для активации чПСК-ДК необходимы молекулярные паттерны, ассоциированные с клеточными повреждениями (DAMPs).

Ключевые слова: дендритные клетки, плюрипотентные стволовые клетки, гемопоэз, дифференциация, раковые клетки

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BSA – bovine serum albumin
cDC1 – conventional DC subset 1
DAMPs – damage-associated molecular patterns
DCs – dendritic cells
ELISA – enzyme-linked immunosorbent assay
GM-CSF – granulocyte-macrophage colony-stimulating factor
hBMP4 – human bone morphogenetic protein 4
hPSC-DCs – human PSC-derived DCs
$hVEGF_{165}$ – human vascular endothelial growth factor 165 kDa
IFNγ – interferon gamma
IL-1β – interleukin beta
IL-4 – interleukin 4
IL-12 – interleukin 12
MLR – mixed lymphocyte reaction
PGE2 – prostaglandin E2
TNFα – tumor necrosis factor alpha

Dendritic cells are professional antigen-presenting cells that play a crucial role in immune response activation and in inducing central and peripheral tolerance [1, 2]. The outstanding ability of DCs to activate peripheral naive T cells entailed the employment of so-called DC vaccines to fight cancer through a tumor-antigen specific action of cytotoxic T cells that were induced by activated DCs. Currently, the primary source of DCs or DC-like cells for the vaccines is peripheral blood monocytes subjected to in vitro differentiation in the presence of GM-CSF and IL-4 [3]. Registered clinical trials, though, revealed that the resulting mono-DCs have low migration capacity and underperform in eliciting strong cytotoxic T-lymphocyte response [4]. An alternative approach is the generation of therapeutic DCs from pluripotent cell sources. Human PSCs that include human embryonic stem cells (hESCs) and human induced PSCs (hiPSCs) have an infinite self-renewal potential while preserving their capacity to develop into virtually any type of cells [5]. This approach's main advantage is that hiPSCs can produce unlimited amounts of autologous DCs to be used in personalized medicine [6]. Similar to deriving DCs from monocytes, the development of DC lineage from hPSCs is induced through the addition of GM-CSF and IL-4 [6].

DCs obtained from hPSCs are essentially immature and require activation, or maturation, to reveal their immune-stimulating properties. Such DCs are refractory to inflammatory activation factors while displaying a phenotype reminiscent of the fetal or neonatal period [7, 8]. Using such cells would be a practical option for the treatment of autoimmune diseases, yet they are not suitable for DC vaccines due to their failure to become activated in response to inflammatory stimuli [9].

This study aims to develop a simple and efficient method allowing massive generation of DCs from hPSCs in defined cell culture conditions, as well as to find effective ways to activate the resulting hPSC-DCs to be further used in the treatment of cancer.

Material and Methods. *Pluripotent stem-cell culture and generation of hPSC-DCs.* The cell lines of H1/WA01 (hESC) and IPS12 (hiPSC) [10] were maintained in an undifferentiated condition on Vitronectin in the mTeSR™1 medium (STEMCELL Technologies, Vancouver, Canada). To initiate hematopoietic differentiation, embryoid bodies (EB) were developed on AggreWell™400 (STEMCELL Technologies) matrices to be further attached to bovine Collagen I (STEMCELL Technologies) in the mTeSR™1 medium containing 10 ng/ml of hBMP4, 50 ng/ml of $hVEGF_{165}$, and 10 μM of Thiazovivin. 4 days later, the medium was replaced entirely with the same volume of StemLine®II HSC expansion medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM of L-glutamine, 0.1 mM of 2-Mercaptoethanol, 0.1 mM of non-essential amino acids, and 50 ng/ml of $hVEGF_{165}$, which was taken away from the medium two days later. On Day 8, hGM-CSF (100 ng/ml) was added to the culture, followed by hIL-4 (100 ng/ml) four days later. From Day 14, half of the DC suspension was collected every two days and pooled into the mTeSR™1 medium, which contained hGM-CSF and hIL-4 at the same concentration. All cytokines, growth factors, and Thiazovivin were from STEMCELL Technologies.

Mixed lymphocyte reaction. Mixed lymphocyte reaction (MLR) was carried out with allogeneic T cells isolated from donor peripheral blood using a Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA). Before the onset of the reaction, the hPSC-DC populations received pre-treatment with mitomycin C. At the end of the reaction conducted in parallel wells for specified periods, T cells were purified and counted on EVE™ Automatic cell counter (NanoEnTek, Seoul, South Korea). The assay was performed in triplicates with the mean for each data point, and the standard deviation was calculated.

ELISA. To measure the secretion of cytokines and growth factors by hPSC-DCs, three-day-old supernatants were purified by high-speed centrifugation, after which respective assays were performed as recommended by the ELISA kit manufacturer (Cloud-Clone Corp., Wuhan, China). All measurements were done at 450 nm on SYNERGY H1 microplate reader (BioTek Instruments, Agilent, Santa Clara, CA).

Real-Time PCR. Total RNA was isolated with an RNA-Extran kit (EX-515-100, Syntol, Moscow, Russian Federation). The first-strand cDNA was synthesized using a reverse transcription kit (OT-1, Syntol). To carry out real-time PCR, a master mix with EvaGreen® dye (R-441, Syntol), as well as specific primers for several DC marker genes, were used, whereas the reactions were carried out on LightCycler 96 (Roche, Basel, Switzerland).

Flow cytometry. For the flow cytometry analysis, cells were harvested, centrifuged, and resuspended in a cold FACS Buffer (1×D-PBS-Ca-Mg, 0.5 % BSA, 2 mM EDTA) at a density of 1×10^6 cells per 100 μL and incubated with Fc-blocking antibodies (Miltenyi Biotec, Germany) for at least 10 min. Further, the cells were incubated on ice with antibodies to various specific antigens for 20 min in the dark. Dead cells were gated out through staining with propidium iodide. All antibodies were obtained from the Abcam (Cambridge, United Kingdom). The flow cytometry was performed on BD FACSCanto II (BD Life Sciences, Franklin Lakes, New Jersey, USA).

Statistics. The statistical analysis relied on the Student's two-tailed test for at least three independent measurements.

Results and Discussion. To initiate the generation of DCs, we employed a protocol for hematopoietic

differentiation of hPSCs in defined conditions and without external cytokine stimulation [11, 12]. This approach offers a close recapitulation of early embryonic development and efficiently generates a wide range of hematopoietic progenitors. The addition of GM-CSF and IL-4 to the hPSC-derived progenitors initiated a massive generation of immature DCs that lost their adherence to the layers of differentiated hPSCs and moved into the culture supernatant starting on Day 14–16 of differentiation (Fig. 1A). Cytokines were added after the first wave of hPSC-derived primitive hematopoiesis started waning. Harvesting DC aliquots went on for several weeks; even after Week 7 of the differentiation, a large number of DCs were still found in the supernatant.

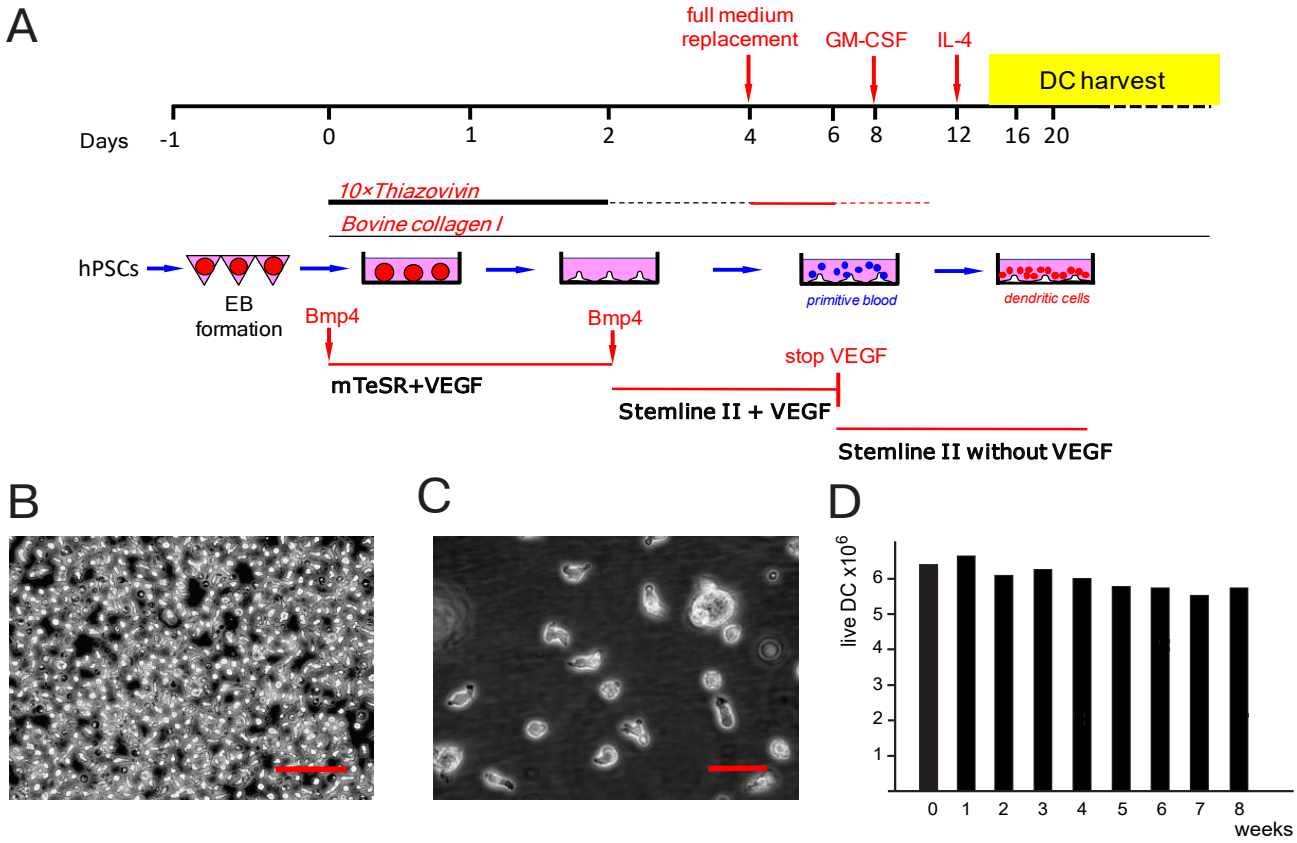

Fig. 1. hPSC-DCs, morphology and stability:
A – a detailed scheme of the DC generation from hPSCs;
B – DCs harvested at day 24 of differentiation in a serum-free medium; scale bar – 100 μm; C – the same cells at a higher magnification, scale bar – 25 μm. A large cell in the upper right corner is a hPSC-derived monocyte differentiating into a macrophage; D – the dynamics of live cells through culturing a cleaned hPSC-DC population

The protocol worked well with the hiPSC line IPS12 and a hESC line H1. The harvested cells featured typical irregular DC morphology (Fig. 1B, C). Relatively small, semi-adherent cells demonstrated significant motility as well as high phagocytic activity, which are features typical of immature DCs. The pooled DC populations were 90–95 % pure, containing low amounts of contaminating monocytes and macrophages (Fig. 1C). Monocytes differentiated into macrophages after a few days in the pooled cultures, whereas the macrophages were quickly removed by replating due to their strong adherence to tissue culture plastic. The number of purified hPSC-DCs following four weeks of harvesting exceeded 5×10^7 cells resulting from the starting hPSC population of 1×10^6 cells. No overt proliferation in the pooled DCs was observed. Yet, apoptosis/necrosis events were minimal, so the numbers of cells in the macrophage-cleaned populations remained relatively stable for at least eight weeks (Fig. 1D). The observations above suggest that hPSC-derived DCs are similar to the steady-state, tissue-resident DCs mediating the fetal homeostasis.

The mixed lymphocyte reaction (MLR) assay demonstrated that hPSC-DCs are capable of inducing proliferation of predominantly $CD4^+$ T helper cells (Fig. 2A and B), which fits well with the functional properties of tolerogenic DCs [13]. Flow cytometry analysis showed a relative homogeneity of the DCs and revealed that only selected DC markers were expressed on the harvested cells (Fig. 2C). This homogeneity of the hPSC-DC population allowed the identification of its phenotype as follows: $CD1a^-CD11b^-CD11^{clow}CD14^-CD45^+CD83^-CD86^+$. Low yet distinct expression of CD11c suggests that the generated cells are lineage-related to the cDC1 subset [1, 14]. The absence of CD11b and CD14 molecules on the cell surface membrane means that the generated DCs do not belong to the monocytic DC lineage. Notable is that one of DC activation markers, CD86, was detected on the DC surface, which was not the case with another activation marker, CD83, reflecting partial activation of hPSC-DCs, which is another feature of tolerogenic DCs [13]. The DCs also proved negative regarding the HLA-DR cell surface expression, the critical activation marker.

To ensure full DC activation, the study involved stimulation with several pro-inflammatory factors. However, adding various combinations of the PGE2 prostaglandin, the IFNγ and IL-1β cytokines, and the TNFα growth factor to the DC culture followed by a two-

day incubation did not have any noticeable impact on the cell phenotype. Moreover, supplementing the activation cocktail with GM-CSF and liposaccharide (LPS) of *E. coli* or *Salmonella enteritidis* produced no measurable effect either. All of the tested cocktails including the factors as mentioned above, too, failed to induce the secretion of IL-12, a primary DC activation indicator (Fig. 3A). Nevertheless, despite the lack of protein products, the pro-inflammatory cocktails were able to induce the transcription of the HLA-DR gene and that of genes encoding the key markers of cells belonging to the human cDC1 subset (Fig. 3B). The presence of HLA-DR transcripts along with the absence of respective molecules on the cell surface further suggests that the hPSC-derived cells correspond to fetal DCs that are functionally similar to tolerogenic ones.

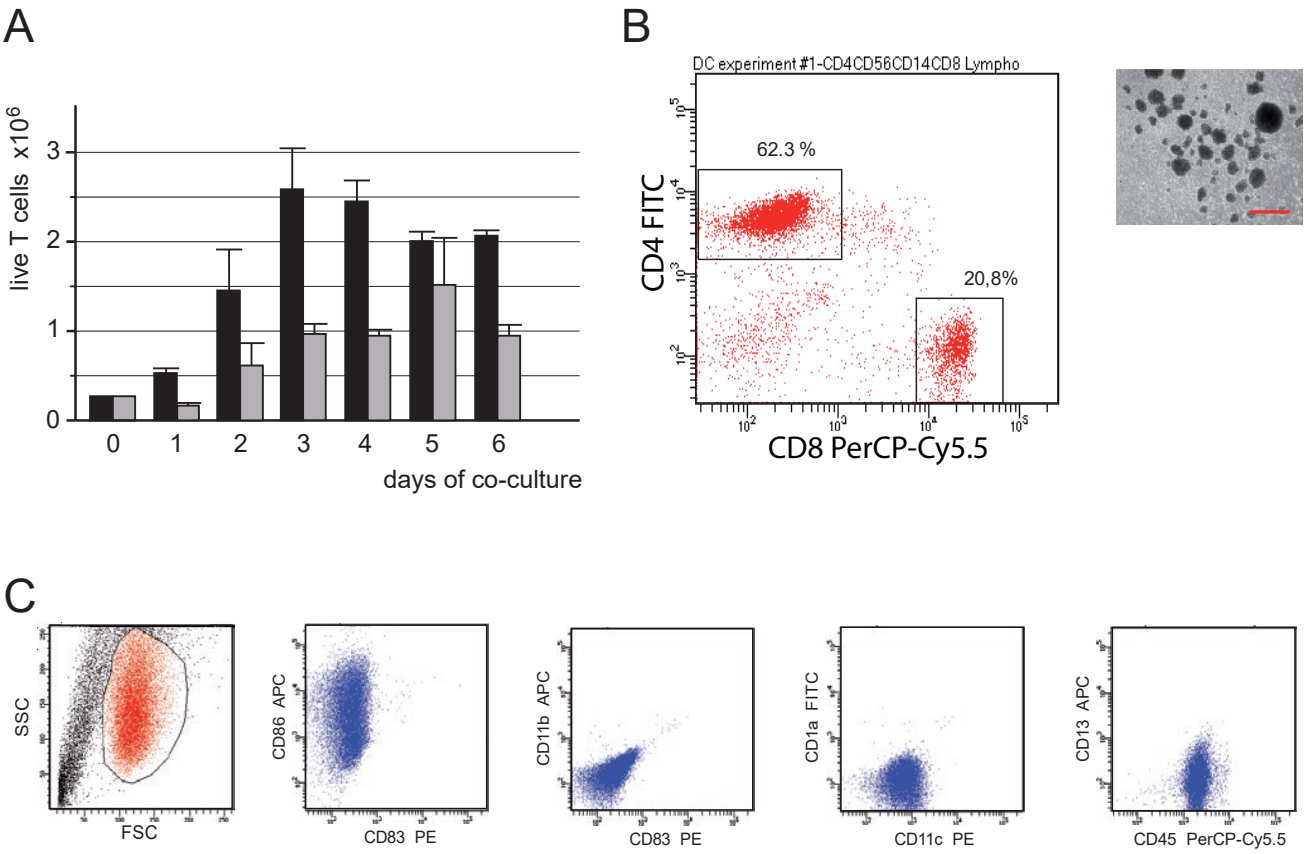

Fig. 2. hPSC-DCs, functional and phenotypic features:
A – mixed lymphocyte reaction (MLR). The black bars reflect the T cell proliferation (2.5×10^5 initial cells) at 1:2 to mixed DCs. The gray bars stand for the ratio of 1:1; B – the flow cytometry plot reveals that most T cells from MLR were $CD4^+$ T helper cells. Inset – strong aggregation of DCs with T cells on 3 days of the co-culture. Scale bar – 500 μm; C – flow cytometry phenotyping of freshly harvested DCs. The selected (gated) area designates the analyzed blast cells – the DC population

The next step was supplementing the pro-inflammatory medium of the cultured hPSC-DCs with thyroid cancer (Bethesda VI type) cell lysates. This treatment entailed partial induction of HLA-DR cell surface expression (Fig. 3C). When we co-cultured the hPSC-DCs with the thyroid cancer cells in the presence of proinflammatory factors for 72 hours, we observed the emergence of a distict DC subpopulation demonstrating a strong HLA-DR cell surface expression (Fig. 3D). It is to be mentioned that the cancer cells are strongly adherent so that pure DCs can be easily isolated for further flow cytometry. This emerging subpopulation is relatively homogeneous and consists of somewhat smaller cells, and has $CD11c^{low}CD45^{low}HLA\text{-}DR^{high}CD14$ phenotype (Fig. 3D). These cells lack the expression of the CD16 and CD66b granulocyte markers while expressing typical DC markers, CD1a, CD1c, and CD11b. The co-culturing also stimulated the production of IL-12 by DCs (Fig. 3E). To conclude, the study shows that despite their fetal phenotype, hPSC-DCs can be efficiently activated via a short-term co-culture with cancer cells.

It must be noted that employing hPSCs in biomedicine will face several issues, which include genomic instability often observed in some hiPSC lines [15], poor quality of the obtained cells, a low yield of end-point cells, and the cost of the technology as a whole. There is yet another issue to be considered, namely, the fetal-like phenotype of hPSC-derived hematopoietic cells, including DCs, which makes these cells hardly suitable for cancer immunotherapy. Nevertheless, the described technical issues cannot dismiss the therapeutic potential of hPSCs.

A pioneering publication once showed that fetal DCs mainly mediate homeostatic immune-suppressive responses during gestation [16]. The fetal DCs, respectively, are not equipped with the molecular tools typical of their adult counterparts and will not respond to conventional activation factors, such as pro-inflammatory cytokines, microbial molecular patterns, and prostaglandin. The suppressive function of fetal DCs brings them close to tolerogenic DCs. It may have a role in quenching the local sterile inflammation that occurs in the early organogenesis due to insufficient macrophage activity involved in cleansing apoptotic and

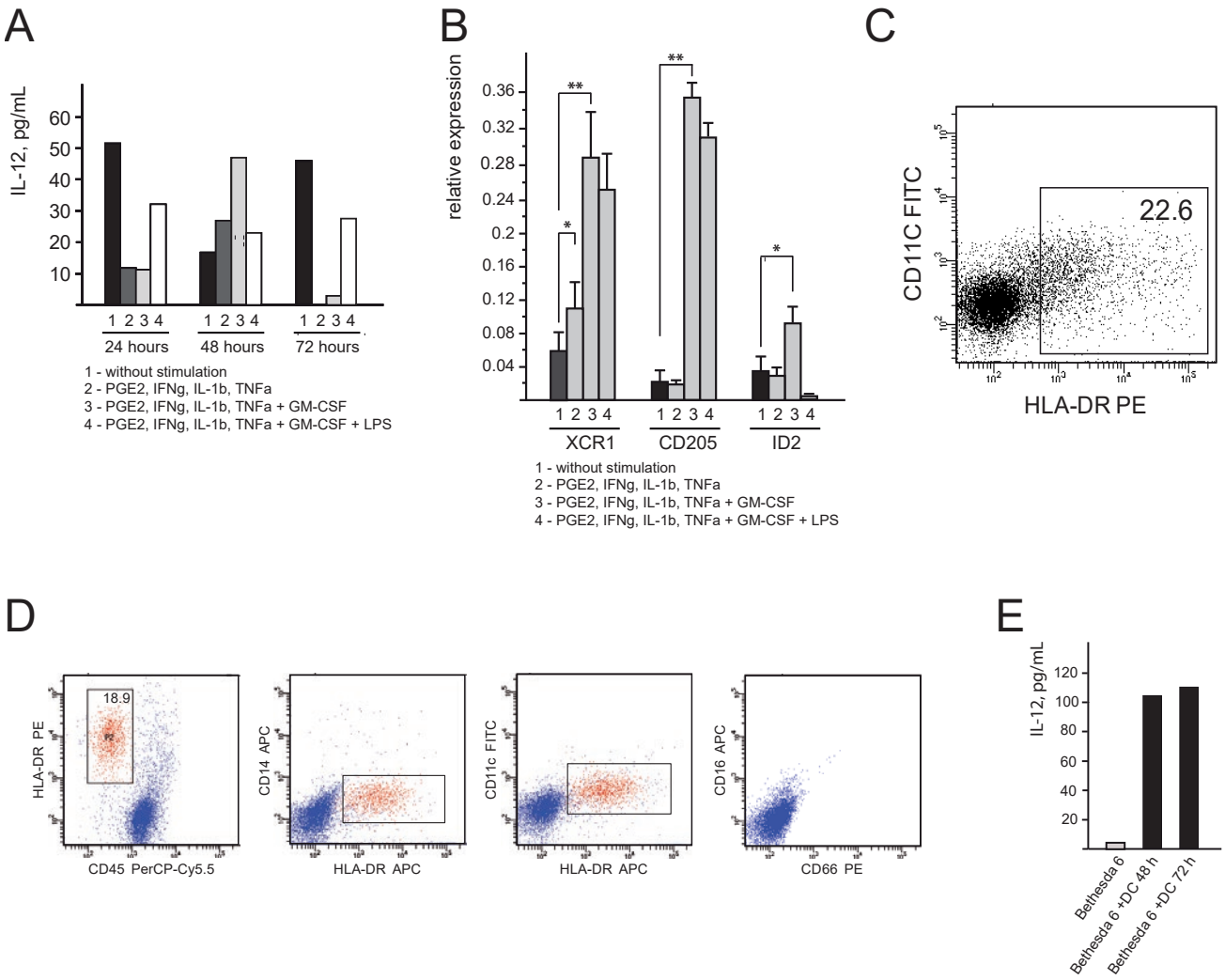

Fig. 3. Activation of the hPSC-DCs:
A – ELISA of IL-12 secretion by DCs treated with various combinations of pro-inflammatory factors;
B – RT-PCR analysis of XCR1, CD205, and ID2 gene expression by dendritic cells treated with various combinations of pro-inflammatory factors. The data is offered as mean±SD, *p<0.05, **p<0.001;
C – moderate upregulation of HLA-DR following loading DCs with cancer cell lysates; D – flow cytometry analysis of hPSC-DCs activated by the cancer cell co-culture. The gate shows the activated $CD45^{low}$ fraction of hPSC-DCs; the co-cultured cells;
E – ELISA of IL-12 secretion by the co-cultured cells

necrotic cells, be they a systemic or an occasional part of embryogenesis [17].

In this work, we managed to routinely generate large amounts of immature DCs from hPSCs in serum-free culture conditions. These hPSC-DCs have been shown to be refractory to activation through the conventional treatment with pro-inflammatory factors, which means they are similar to fetal DCs, which are not primed to respond to inflammatory activation due to strong suppression of inflammation in the developing fetus [16]. Seeking to find a way to ensure effective activation of hPSC-DCs, we cultured them with cancer cells to see that that short-term co-culture facilitated strong activation of the hPSC-DCs subpopulation. This effect must be mediated by DAMPs accumulating in the cancer cell culture due to occasional cell necrosis.

Conclusion. The obtained outcomes suggest that the study selected proper conditions for the massive generation of hPSC-DCs in a serum-free medium. This means that the respective method is suitable for therapeutic applications. Given their resistance to inflammatory stimuli, proof has been obtained that the DCs are fetal. Another finding was that loading hPSC-DCs with oncoantigens at a short-term co-culture with cancer cells resulted in a robust activation in a subset of the DCs. Our work supports the idea of using pluripotent cell sources for making DCs that can be employed in cancer immunothreapy.

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