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Anti-Tumoral Effects of Natural Killer Cells Differentiated *In Vitro* from Cord-Blood Hematopoietic Stem Cells on Umbilical Cord Mesenchymal Stem Cells against Glioblastoma

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Abstract

Purpose: Glioblastoma is the most malignant primary adult brain tumor with a poor prognosis; therefore, novel therapies are needed. Natural Killer (NK) cells are promising candidates for immunotherapy because of their ability to eliminate tumor cells without prior sensitization. An inadequate number of NK cells remain a major obstacle. The primary purpose of this study was to develop NK cells from Cord Blood Hematopoietic Stem Cells (CB-HSCs) on Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) feeder layer and analyse cytotoxic potential of NK cells against glioblastoma *in vitro*.

Methods: UC-MSCs were treated with mitomycin-C to be used as a feeder layer. CB-HSCs were co-cultured with UC-MSCs and differentiated into NK cells by using medium containing cytokines such as thrombopoietin, Flt3-ligand, stem cell factor, IL-6, IL-7, IL-15 and IL-2. NK cells were characterized by immunocytochemistry. The *in vitro* cytotoxic effect of NK cells on T98-glioblastoma cells was determined by annexin V-fluorescein isothiocyanate/propidium iodide staining followed by flow cytometric analysis. qRT-PCR was performed to measure gene expression levels of *KRAS*, *TP53*, *TGFBR2* and *NANOG* in glioblastoma cells after treatment with NK cells.

Results: NK cells were successfully differentiated from CB-HSCs on the UC-MSC feeder layer with the strong expression of cytotoxic receptors after 6 weeks. They demonstrated potent cytotoxicity against glioblastoma *in vitro*. Additionally, the *KRAS* oncogene expression in glioblastoma cells decreased upon co-culture with NK cells.

Conclusion: NK cells differentiated from CB-HSCs on UC-MSC feeder-layer were capable of eliminating glioblastoma cells *via* apoptosis *in vitro* and warrant further investigation *in vivo* and clinical settings.

Keywords: Apoptosis; Tissues; Monocyte Chemo Attractant Protein; Brain tumor; Cancer; Immunotherapy

Abbreviations: CB: Cord Blood; CD: Cluster of Differentiation; HSC: Hematopoietic Stem Cell; IL: Interleukin; MSC: Mesenchymal Stem Cell; NK: Natural Killer; UC: Umbilical Cord

Introduction

Glioblastoma (formerly known as Glioblastoma Multiforme, GBM) is the most common and most malignant primary adult brain tumor. This aggressive behaviour of this cancer correlates with its poor survival rates. The median survival is 15 months, and the 5 year survival rate is 4% to 5% despite the current treatment options such as surgery, radiotherapy, and chemotherapy [1-3]. One of the trending treatment modalities in cancer therapeutics is immunotherapy, particularly adoptive cell therapies. In these cell-based immunotherapies, Natural Killer (NK) cell-based therapy has been widely accepted as a good candidate because of its critical role in tumor elimination [4,5]. Mature NK cells fundamentally eliminate cancer cells with diminished or absent expression of antigen-presenting molecules known as Major Histocompatibility Complexes (MHCs) on the cell surface. When an MHC molecule binds to Killer cell Immunoglobulin-like Receptors (KIR) on the NK cell surface, KIR sends inhibitory signals. This inhibition protects MHC expressing healthy cells from targeting by NK cells. During the maturation process, NK cells need this interaction and become "licensed" to use their weapons to kill target cells [6,7]. After maturation, they are susceptible to inhibition by MHC molecules. Favourably, tumor cells down regulate their MHC molecules to evade other immune cells such as T-cells, thus becoming a target for mature NK cells [8-10]. Moreover, due to cellular stress and DNA damage during rapid proliferation, tumor cells express molecules also recognized by NK cells *via* various surface receptors. These receptors on mature NK cells, such as the NKG2D receptor, bind MHC class I polypeptide-related sequence A MICA, MICB, and UL16-binding proteins, and send activation signals. If the activation signals are strong enough, they can counteract inhibitory signals and initiate innate and adaptive immune responses [11-13]. They attack the tumor cells by releasing cytotoxic granules called perforin and granzyme, thus

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inducing Caspase activated apoptosis of tumor cells. Additionally, NK cells secrete interferon-γ and tumor-necrosis factor-α that modulate immune responses [12,14]. NK cells originate from hematopoietic stem cell progenitors in the bone marrow and are characterized by their CD56+/CD16+/CD3- surface markers [15]. However, the development of NK cell-based immunotherapy has practical difficulties, such as producing sufficient, highly purified, and activated cells [16]. Most studies using NK cells as adoptive immunotherapy mainly investigated obtaining NK cells from peripheral blood, haploidentical donors, or commercially available KIR-negative NK cell line, NK-92, with limited results [17-19]. A further approach was to produce and expand NK cells ex-*vivo*, enabling manipulations or after infusion of manufactured NK cells, which yielded more satisfactory results [20-22].

For these reasons, we here focused on obtaining NK cells by differentiating Cord Blood Hematopoietic Stem Cells (CB-HSC) ex-*vivo*. Most of the umbilical cord is discarded after birth, although it possesses a rich source of stem cells. The umbilical cord has two main components: Cord blood and the other is the Wharton's jelly which is the supporting mesenchymal tissue around vessels. Cord blood is a rich source of HSCs that can be differentiated into NK cells after induction by various cytokines to commit to the lymphoid progenitor [23]. Feeder layer cell systems can be used further to support the commitment and expansion of these cells. Mesenchymal stromal cells, such as bone marrow stromal cells, are commonly used as a feeder layer for HSC differentiation and expansion to mimic their physiological supporting properties during haematopoiesis. They secrete valuable cytokines such as IL-6, IL-8, Monocyte Chemo Attractant Protein 1 (MCP1), G-CSF, Growth-Related Oncogene (*GRO*), Tissue Inhibitor of Metalloproteinase (TIMP)-1, and TIMP-2, which help to maintain HSC proliferation and stem ness [24]. Compared with the bone marrow MSCs, UC-MSCs have been shown to express a similar cytokine secretion profile [25]. While being superior in expansion properties and easier to harvest [26]. UC-MSCs also promote the expansion of HSCs synergistically with cytokines ex *vivo* [27,28].

Here, we report the first *in vitro* study showing NK cells could be obtained by differentiating CB-HSCs on the UC-MSCs feeder layer in the presence of cytokines relevant to NK cell proliferation and differentiation without impacting their cytotoxic potential. Additionally, we demonstrated the effect of NK cells' cytotoxicity against GBM *in vitro* and the expression of several tumor-associated and cancer stem ness associated genes in the NK cell-treated T98 glioblastoma cell line.

Materials and Methods

Cell cultures

UC-MSCs and CB-HSCs were obtained from Erciyes University Genome and Stem Cell Center, Kayseri, Turkey. The cells were thawed and transferred into T25 culture flasks at a density of 1×10^3 cell/ml. Fifth passage of UC-MSCs were cultured in α-MEM (Sigma- Aldric, USA) supplemented with 15% FBS (Gibco, 10270-106) 100 U/ml penicillin and 100 µg/ml streptomycin (BI, Cromwell, CT, USA), 2 mmol/l L-glutamine (Capricorn, GLN-B), and 10 ng/ml basic Fibroblast Growth Factor (bFGF) (Prospec-cyt-557-b). CB-HSCs were cultured in α-MEM supplemented with 10% FBS, 10 ng/ml bFGF and 100 U/ml penicillin and 100 µg/ml streptomycin. T98G glioblastoma cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) (High Glucose) supplemented with 10% FBS, 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 µg/ml streptomycin. All the cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2-3 days. The cells were sub-cultured for further experiments when they reached to ~80% confluence.

UC-MSC feeder cell preparation and Mitomycin-C treatment

UC-MSCs (1×10^3 cells/well) were plated in a 6-well culture plate with α-MEM basal medium supplemented with 15% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and 10 ng bFGF. Medium was changed every two days until the cell confluency reached 80%-85% confluence. After reaching confluency, the proliferation of the UC-MSCs was stopped by adding 20 µl/ml Mitomycin-C and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for two hours. The cells were then washed with sterile PBS (Biosera, LM-S2042%500) and the culture medium was changed for co-culturation.

Differentiation of NK cells from CB-HSCS on UC-MSC-feeder-layer

In insert co-culture systems, two cell populations that are co-cultured indifferent compartments (insert and well) stay physically separated, but may communicate *via* paracrine signaling through the pores of the membrane. Here, CB- HSCs (1×10^3 cell/ml) were seeded on the inserts then; the inserts were placed onto the 6-well culture plates that were seeded with the previously mentioned mitomycin-treated UC-MSCs at 1:1 ratio. CB-HSCs were incubated in Stem line Hematopoietic Stem Cell Expansion Medium (Sigma, USA) supplemented with 50 ng/ml stem cell factor (SCF), 50 ng/ml Flt3 ligand, 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF), 10 ng/mg Thrombopoietin (TPO), 20 ng/ml IL-6 and 20 ng/ml IL-7 for nine days (Sigma, USA). After the first nine days induction period, TPO was replaced with 40 ng/ml IL-15 and the cells were incubated for another five days. On day 14 of the incubation, Flt-3 ligand was replaced with 12.5 ng/ml IL-2. The total incubation period lasted 42 days. Differentiation was observed and photographed *via* an inverted microscope (IX71, Olympus, Japan).

Indirect immunocytochemical analysis of the NK cells differentiated from CB-HSCs

NK cells were harvested on day 42. Immunocytochemistry was performed using antibodies against one hematopoietic and one mesenchymal stem cell marker (CD34 and CD90, respectively), as well as NK cell surface markers (NCAM, NKG2D and NCR1). Cells were fixed with 4% paraformaldehyde solution for 30 min. After washing with PBS, they were permeabilized with 0.1% Triton X-100 (AppliChem, A4975, 0100) in PBS solution for 15-min on ice. Endogenous peroxidase activity was blocked with hydrogen peroxide solution for 10 min, they were then washed with PBS. The cells were incubated with blocking solution (Invitrogen, CA, USA) to reduce nonspecific binding for one hour at room temperature. They were then incubated with the following primary antibodies: mouse monoclonal anti-CD34 (1:50 dilution, sc 74499, Santa Cruz Biotechnology, USA), mouse monoclonal anti-CD90 (1:50 dilution, sc53456; Santa Cruz Biotechnology, USA), mouse monoclonal anti-NCAM (1:50 dilution, Abcam, ab9018), rabbit polyclonal anti-NKG2D (1:100 dilution, ab203353; Abcam), rabbit polyclonal anti-NCR1 (1:50 dilution, Abcam, ab199128). They were washed three times for 5 min in PBS and incubated for 30 min with biotinylated secondary goat anti-mouse antibody at room temperature as instructed by the manufacturer. After washing with PBS, streptavidin peroxidase was added and incubated for 30 min. After washing with PBS, DAB (3,3′-diaminobenzidine tetrahydrochloride) (ScyTek, UT, USA) was applied for 5 min for immunoreactivity detection. All cells

were washed with distilled water and counterstaining was performed with Mayer's hematoxylin and they were then washed again and mounted with mounting medium (Spring Bioscience, DMM-125). The intensity of immunolabeling was evaluated by the two investigators at different times with light microscopy (BX40, Olympus, Tokyo, Japan). The immunoreactivities were considered as negative (-), weak (+), moderate (++) and strong (+++).

T98G glioblastoma cell culture

T98G Glioblastoma cells (ATCC; Manassas, VA) cultured with DMEM (High Glucose) supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Medium was changed every two days until the cell confluency reached 80%-85% confluency.

T98G Glioblastoma and NK cells co-culture

T98G Glioblastoma cells were co-cultured with the NK cells differentiated from CB-HSCs in a 6-well plates as indicated in Table 1. Cells were incubated in DMEM (High Glucose) supplemented with 10% FBS, 2 mmol/l L-glutamine and 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2. Trans-well co-culture systems were terminated on the fifth, tenth and fifteenth days. Glioblastoma cells were collected from 6-well plate for Annexin V assay on days 5, 10, and 15 and qRT-PCR on days 10 and 15 with Effector-Target (ET) ratio of 1:1, 2:1, and 4:1 respectivel.

	Day 5	Day 10	Day 15
T98G Glioblastoma cells	4 × 10^3 cells/ml	2 × 10^3 cells/ml	1 × 10^3 cells/ml
NK cells	4 × 10^3 cells/ml	4 × 10^3 cells/ml	4 × 10^3 cells/ml

Table 1: T98G Glioblastoma and CB-HSC-NK cells co-culture.

Flow cytometric analysis of TG98 glioblastoma cells

To detect cell death, Annexin V/PI method was used in flow cytometric analyses. T98G glioblastoma cells were collected and centrifuged on the fifth, tenth and fifteenth days of co-culture. The collected glioblastoma cells treated with NK cells were subject to Annexin V/PI Apoptosis kit protocol (BD Pharmingen). Cells were resuspended in 100 µl of 1X Binding Buffer. 5 µl of Fluorescein Isothiocyanate (FITC)-Annexin V (component no. 51-65874X) and 5 µl of Propidium Iodide (PI) (component no. 51-66211E) were added. Then, the cells were incubated in dark for 5-10 min at room temperature. Lastly, 400 µl of 1 × Binding Buffer was added to each tube. The fluorescence intensity was measured using a BD Bioscience Acuri 6 flow cytometer. (FL1 channel for FITCH-Annexin-V: Ex=485 nm; Em=535 nm, FL2 channel for PI: Ex=535 nm; Em=617 nm). Cells treated with nothing served as the control group.

qRT-PCR analysis

The determination of gene expression levels was performed using quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from T98G Glioblastoma cells on the tenth and fifteenth days using the Rneasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the VitaScript™ FirstStrand cDNA synthesis kit (Procomcure Biotech), and cDNA was obtained for the qRT-PCR reactions. 2 µl of cDNA was amplified using the 10 µl 2X Magic SYBR Mix (Procomcure Biotech) containing 0.2 µM of the primers. The total volume of each reaction was 20 µL. The primer sequences (forward and reverse) for *KRAS*, *TP53*, *TGFBR* and *NANOG* are listed in Table 2. The relative expression of the individual gene was calculated using the comparative cycle threshold (2-ΔΔCt) method. Sample Ct-values were normalized by the Ct-values of the housekeeping gene. In the T98G glioblastoma cells, beta-actin was the reference housekeeping gene used for quantification.

Target genes	Forward primer sequence	Reverse primer sequence
Beta-actin	5'-CTGGAACGG TGAAGGTGACA-3'	5'-AAGGGACTTCC TGTAACAATGCA-3'
KRAS	5'-GACTCTGAAGATG TACCTATGGTCCTA-3'	5'-CATCATCAACA CCCTGTCTTGTC-3'
TP53	5'-TCAGATAGC GATGGTCTGGC-3'	5'-CACGCACCT CAAAGCTGTTC-3'
TGFRB2	5'-ATGACATCTC GCTGTAATGC-3'	5'-GGATGCCCT GGTGGTTGA-3'
NANOG	5'-TCTCCAACATC CTGAACCTCA-3'	5'-TTGCTATTC GGCCAGTT-3'

Table 2: The PCR primers sequences used for *KRAS*, *TP53*, *TGFRB2* and *NANOG* genes and for the housekeeping gene beta-actin.

Statistical analysis

The relative expression of the target gene was analyzed *via* the Qiagen Gene Globe Data Analysis Center/PCR/ gene expression analysis system. The student's t-test was used to evaluate statistically significant differences using the Prism 8 software (GraphPad, USA) and the $p<0.05$ level was considered as statistically significant.

Results

In vitro differentiation of CB-HSCs into NK cells on UC-MSC feeder layer

The morphology of CB-HSCs cells appears as bright and round cells showing non-adherence. CB-HSCs in suspension were used for co-culture and NK-cell differentiation on day 7. Untreated UC-MSCs appear as large, flat fibroblast-like morphology and adhere to the surface of the wells to form a monolayer. Mitomycin-C treatment had no significant effect on the UC-MSC morphology. During the co-culturation and differentiation processes, developing NK cells became brighter, denser and slightly smaller in size, and changed their expression of surface markers (Figures 1-3).

Representative phase-contrast images showed that after 42 days, UC-MSCs promoted the expansion and differentiation of CB-HSCs to NK cells.

Immunocytochemical characterization of NK cell differentiation from CB-HSCs

To evaluate whether differentiated cells expressed NK cell markers or stem cell markers, we performed immunocytochemistry and stained NK cells differentiated from CB-HSCs with antibodies against NKG2D (CD314), NCAM1, and NCR1(NKp46) as NK cell markers, CD34 as hematopoietic stem cell marker, and CD90 as a mesenchymal stem cell marker. NCAM1 immunoreactivity was weakly positive (Figure 3) while NKG2D and NCR1 immunoreactivities were strongly positive in CB-HSC-NK cells. (Figures 3A-3C). CB-HSC-NK cells did not exhibit detectable immunoreactivity for CD34 and CD90, as expected (Figures

3E and 3F respectively). NKG2D and NCR1 are two major activating receptors responsible for NK cell cytotoxicity. The strong positivity of these two markers and the negativity of stem cell markers suggest that CB-HSC-NK cells have cytotoxic capacity and are fully differentiated. Control staining was negative (Figure 3A).

Flow cytometry resulting of annexin V-FITC-PI stained T98G glioblastoma cells after treatment with NK cells differentiated from CB-HSCS

Since the cytotoxic effect of NK cells is related to the induction of apoptosis, we have examined the flow cytometry pattern of Annexin V-FITC stained T98G cells after co-culture with CB-HSC-NK cells form 6-well plate. Cells positive for Annexin V-FITC and negative for Propidium Iodide (PI) are in the early stage of apoptosis as shown in the Lower-Right (LR) quadrant, while cells positive for both Annexin V-FITC and PI are in the late stage of apoptosis or necrosis as shown in Upper-Right (UR) quadrant (Figure 4). Thus, the degree of apoptosis correlates with the number of positive Annexin V-FITC cells. The results showed that T98G cells co-cultured with CB-HSC-NK cells could bind Annexin V-FITC and therefore CB- HSC-NK cells can induce apoptosis (39.5%, 6.3%, 13.4%; percentage of apoptotic cells on day 5, day 10, and day 15, respectively.). The proportion of Annexin V-FITC-positive cells decreased after day 5 because of the decreasing number of NK cells in the absence of interleukins essential for NK cell survival. These results demonstrate that NK cells differentiated from CB-HSCs possess cytotoxic and antitumoral properties against T98G glioblastoma cells.

T98G glioblastoma and CB-HSC-NK cells were co-cultured for a maximum of 15 days in the absence of interleukins. The induction of apoptosis was evaluated by Annexin V-FITC and Propidium Iodide (PI) staining on day 5, day 10, and day 15.

Changes in gene expression of T98G Glioblastoma cells after treatment with NK cell differentiation from CB-HSCs

The interaction between cancer cells and the immune system extends beyond the cellular level, immune cells can alter the gene expression levels of cancer cells by inducing evolutionary selection or interaction at the cytokine and molecular level. How this cytolytic activity affects oncogenic and resistance genes in tumors is still remains to be elucidated [29,30]. To evaluate the effect of CB-HSC-NK cells on expression of *TP53*, *KRAS*, *TGFBR2*, and *NANOG* genes in T98G glioblastoma cell line, qRT–PCR was performed. PCR results showed that although NK cells do not affect *TP53* and *NANOG* expression levels significantly, they decrease the expression of *KRAS* in glioblastoma on days 10 and day 15 of culturation (Figure 5).

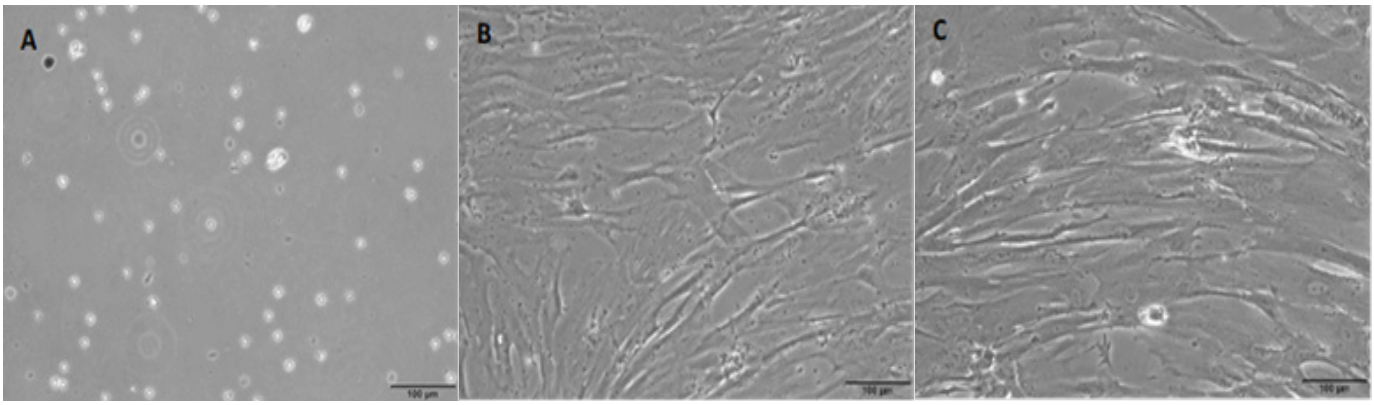

Figure 1: A) Cellular morphology of undifferentiated cord blood hematopoietic stem cells; B) Umbilical cord mesenchymal stem cells; C) Umbilical cord mesenchymal stem cells after mitomycin-c treatment. (Scale bar=100 μm).

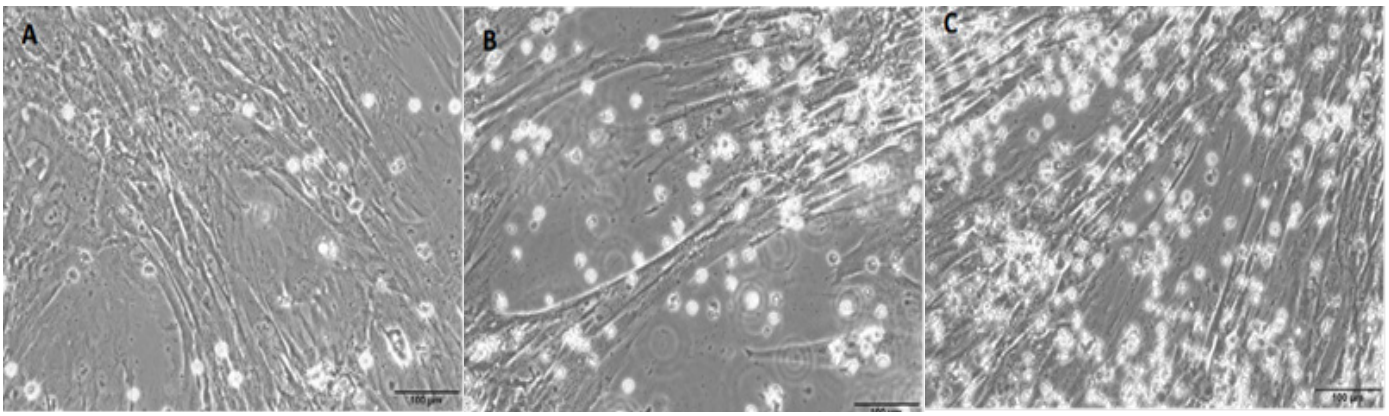

Figure 2: Cellular morphology of the Cord Blood Hematopoietic Stem Cells co-cultured on umbilical cord mesenchymal stem cells as the feeder layer in the presence of cytokines at (A) 9 days, (B) 14 days, and (C) 42 days. (Scale bar=100 μm).

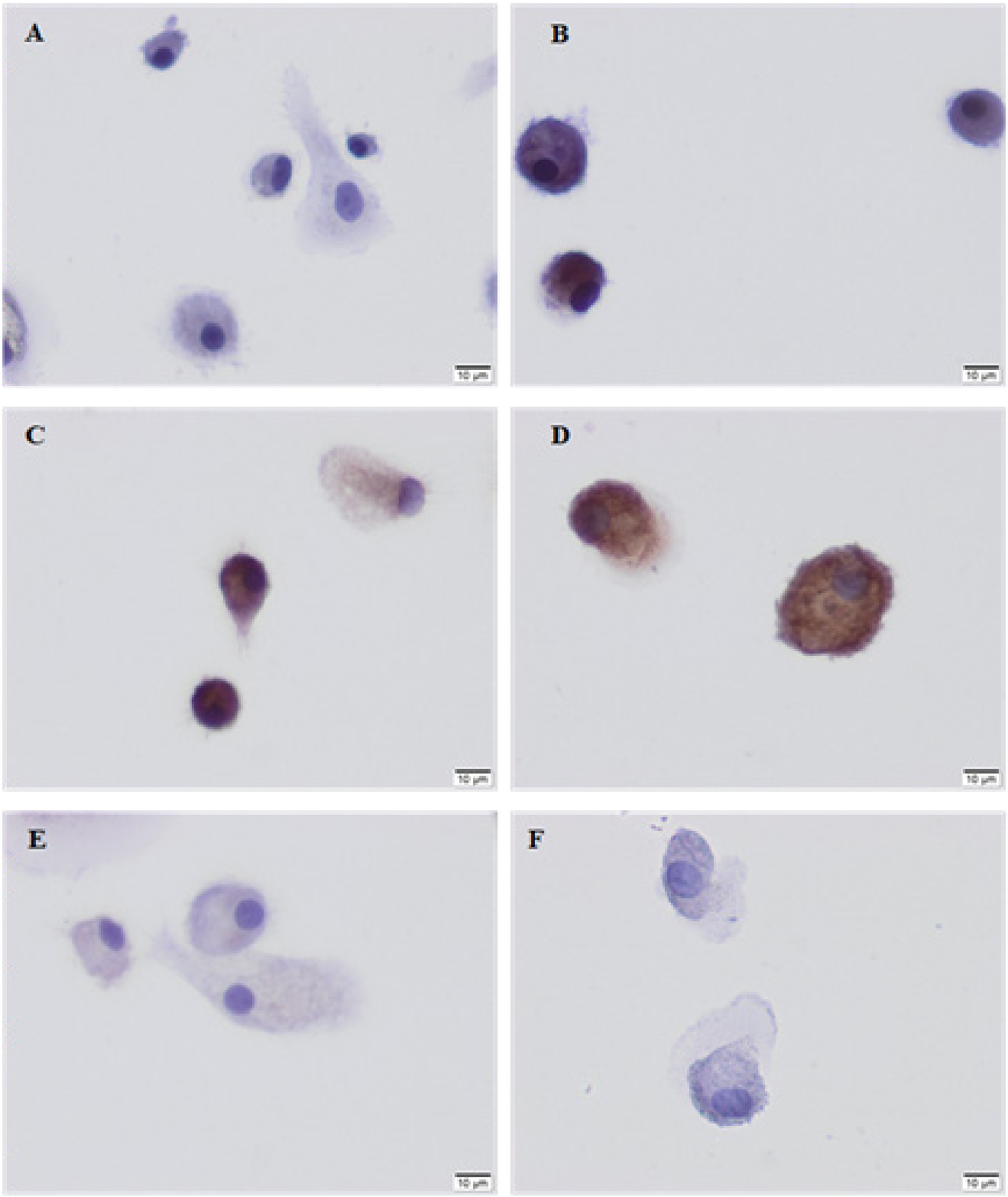

Figure 3: Control (A) *NCAM1*, (B) NKG2D, (C) *NCR1*, (D) *CD34*, (E) CD90, (F) Staining in CB-HSC-NK cells. Scale bars: 10 µm. *NCAM1*: Neural Cell Adhesion Molecule 1; NCR1: Natural Cytotoxicity Triggering Receptor 1; NKG2D: Natural Killer Group 2D; CD: Cluster of Differentiation flow cytometry results of annexin V-FITC-PI stained T98G glioblastoma cells after treatment with NK cells differentiated from CB-HSCS.

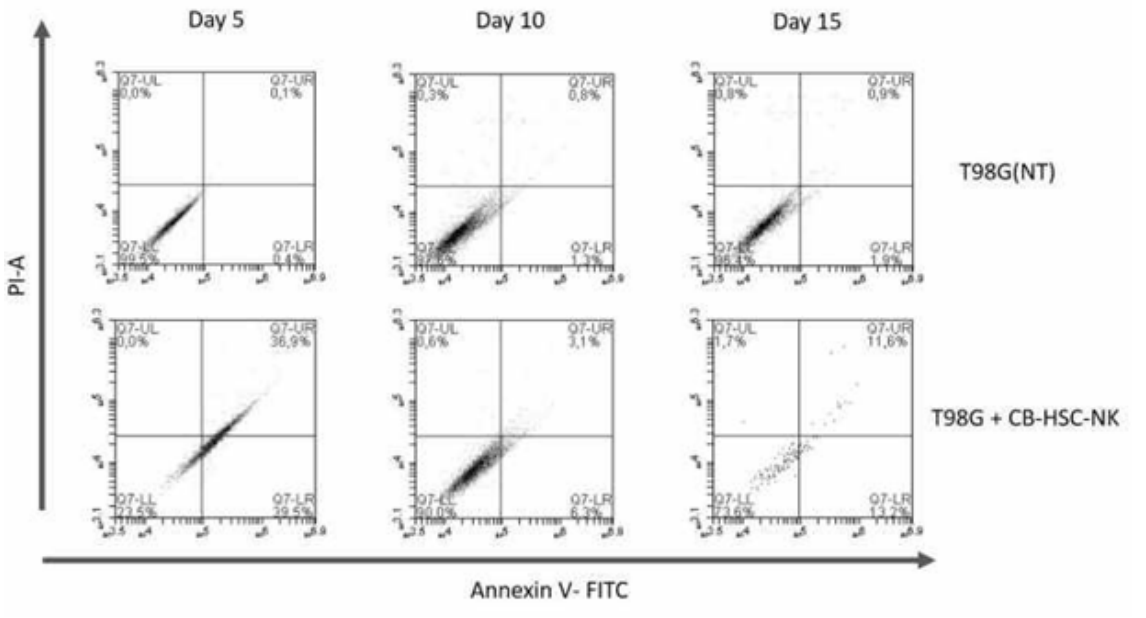

Figure 4: Effect of NK cells differentiated from cord blood hematopoietic stem cells on the induction of apoptosis of T98G glioblastoma cells.

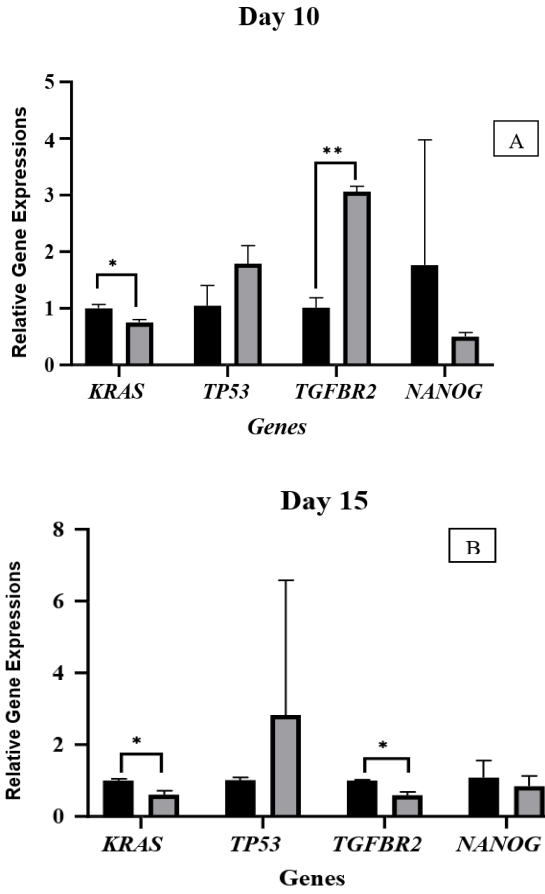

Figure 5: Variations in gene expression of *KRAS*, *TP53*, *TGFBR2* and *NANOG* genes in T98G cells after co-cultured with CB-HSC-NK cells compared to the T98G cells without co-culturing as the control group on (A) Day 10, and (B) Day 15. **Note:** Control; T98G+CB-HSC-NK; **p<0,01, ***p<0,001 T98G+CB-HSC-N..

T98G glioblastoma and CB-HSC-NK cells were co-cultured for a maximum of 15 days in the absence of interleukins. The induction of apoptosis was evaluated by Annexin V-FITC and Propidium Iodide (PI) staining on day 5, day 10, and day 15.

Discussion

More than 250 000 people are affected by primary malignant brain tumors annually worldwide. Unfortunately, the vast majority of malignant brain tumors are glioblastoma which is the most aggressive brain tumor. Despite advances in anti-tumor therapy, patients' outcomes remain poor due to inevitable recurrence and malignant progression [31].

Over the last two decades, cancer immunotherapy has gained increasing interest and attention in the literature. Especially, adoptive cell therapy has been an attractive modality because of its promising antitumoral effects in both *in vivo* and *in vitro* studies. In the adoptive cell therapy, many immune cells, such as dendritic cells, T cells, and Natural Killer Cells (NK cells), have been investigated as candidates. Among them, NK cells were the most advantageous due to their antigen-independent cancer cell recognition; therefore, no need for prior sensitization. Although NK cells have been shown to be effective, one of the major limiting factors for NK cell therapy is insufficient production. To overcome this obstacle, we tested an alternative method to obtain NK cells by differentiating Cord Blood-Hematopoietic Stem Cells (CB-HSCs) on umbilical cord-mesenchymal stem cells (UC-MSC) feeder layer. An umbilical cord is often discarded as a waste material after the delivery. Using both the stem cell classes found together in the umbilical cord, we intended to repurpose the umbilical cord to be used as a unique source for NK cells. In a similar approach, Kao showed that expanded cells could be differentiated into cytotoxic NK cells after five weeks of incubation in a cytokine cocktail consisting of IL-2, IL-7, IL-15, SCF, and Flt3-Ligand, L-glutamine, FBS [32]. Even though this method has proved CB-HSCs could be a promising source for immunotherapy, the number of NK cells obtained was suboptimal. Boissel performed a two-step approach to obtain a higher number of NK cells. First, they depleted CD3+ lymphocytes from Cord Blood Mononuclear Cells (CB-MNCs) by immunomagnetic bead selection. Second, they co-cultured CD3- depleted CB-MNCs with a gamma irradiated-fibroblast feeder layer derived from Wharton's jelly of the umbilical cord in the presence of different concentrations of cytokines for two weeks. Using this method, they obtained a significant expansion of NK cells [33]. Most other studies also suggest that the interaction between NK and stromal cells increases the expansion as well as the maturation of NK cells; therefore, this situation indicates the need for a microenvironment supported by stromal cells [34-37]. In previous studies, various attempts have been made to stimulate the NK cell expansion with irradiated UC-MSCs. However, the irradiation resulted in a decrease in the number of UC-MSCs due to accelerated cell death [33,38]. Additionally, one of the problems of using UC-MSCs as a feeder layer is the lack of a standardized isolation method and characterization that can lead to inconsistent results [39].

In our study, we pursued a combined approach to harness the differentiation-inducing and proliferative effects of the cytokines released from the UC-MSCs in both the early and late stages of NK cell differentiation from CB-HSCs. We applied a protocol to induce differentiation and expansion of CB-HSCs to NK-cells by co-culturing with mitomycin-C-treated UC-MSC-derived feeder cells in the presence of several cytokines for six weeks. The use of mitomycin-C to inhibit the proliferation of UC-MSCs did not result in cell death and maintained viability until the final day of the experiment. At the end of our protocol, we achieved fully differentiated NK cells that lack stem cell markers and express activating receptors. Notably, our differentiated NK cells showed strongly stained the activating receptor NKG2D, a key mediator of NK-cell target recognition, and NCR1 (NK p46), a cytotoxicity receptor that is highly specific for NK cells [40,41]. NK p46 is also involved in target recognition *via* still-unidentified ligands and is found on all mature NK cells [42,43]. Although our results were encouraging, the length of our experiment was longer than the previous studies.

After characterization, the CB-HSC-NK cells were then assessed for their cytolytic activity against T98G glioblastoma cells. Although it is controversial and depends on the patient population, glioblastoma cells evade the immune system by poor expression of MHC I molecules and ligands for NK cell activating receptors [44,45]. The amount of the activating receptors on the NK cells may play a more critical role than MHC I expression level on glioma cells in determining susceptibility to NK cell lysis. It has been shown that cytokine stimulation of the NK cells is needed for a more potent anti-tumoral effect, and cytokine- activated NK cells that express high levels of NKp46 and NKG2D receptor represent a suitable candidate for glioblastoma immunotherapy [46,47]. Consistent with other studies, the cytokines used in cell culture, as well as the other cytokines released by UC-MSCs, primed the CB-HSC-NK cells to achieve cytotoxic potential against T98G glioblastoma cells. In spite of this, after day five, the cytotoxic potential has dramatically declined due to the absence of the essential cytokines.

The significant tumor-promoting role of the *KRAS* pathway has been well established in pancreatic and lung cancers, while its significance in glioblastoma is gaining momentum. Holmen have previously shown that glioblastoma cells require *KRAS* signaling to maintain tumorigenesis, and suppression of the Ras pathway results in apoptosis and tumor regression [48]. In the present study, we observed that NK cells suppress *KRAS* expression, although the exact mechanisms of how NK cells down regulate *KRAS* require further investigation. *P53* and *TGFβ* related genes and pathways are also deregulated in glioblastoma [49,50]. *TP53* acts as a tumor-suppressor gene in physiological conditions. In many cancers, mutations in the *TP53* gene contribute significantly to tumor progression and treatment resistance by gaining new functions and losing their regulatory role in apoptosis [51]. The restoration of functional *p53* in tumor cells is being investigated widely in the literature. Likewise, Transforming Growth Factor-β Receptor Type 2 (*TGFBR2*), A Trans membrane protein with serine/threonine kinase activity, plays a complex role in tumor development. [52]. *TGFBR2* is expressed higher in glioma cells than the normal brain tissue, and its inhibition has been proposed as a promising therapeutic target. *TGFβ* binds to *TGFBR2*, Trans activates the *TGFBR1*, and initiates a molecular cascade that contributes to angiogenesis, invasion, and proliferation of glioma. Interestingly, it has been shown that *TGFβ* can act both as a tumor suppressor and promoter depending on the stage of oncogenic transformation [53,54]. This particular characteristic of this gene makes it difficult to determine the net effects of NK cells on *TGFBR2* gene expression.

Even though the appropriate therapeutics are administered and eliminate most cancer cells, a subset of tumor cells may restore the tumor cell population and lead to recurrence. These cells are known as Cancer Stem Cells (CSCs). CSCs possess stem cell-like characteristics, such as self-renewal and proliferation. Therefore, it is crucial to target CSCs to achieve a better treatment response. One of the genes responsible for cancer stemness is the *NANOG* gene. It encodes a

transcription factor that binds to DNA and activates the genes involved in self-renewal, thus providing pluripotency [55,56]. Interestingly, it has been shown that cancer cells can evade NK cell attack due to *NANOG*-mediated suppression of intercellular adhesion molecule-1 [57]. Ye demonstrated that LN229 and GBM2 glioblastoma cell lines increase their malignant potential such as migration, proliferation, and invasion by lent viral transduction of *NANOG* overexpression [58]. Even though the expressions of other stemness markers, such as CD44 are observed in the T98G cell line, *NANOG* gene expression in the T98G cell line is not widely studied [59]. The Cancer Dependency Map (DepMap) portal website (https://depmap.org/portal), which uses genome-wide CRISPR and shRNA screens, can be used to provide the gene effect information for each gene in cancer cell lines. By comparing *NANOG* gene effect scores (Chronos) of LN229 (-0.035) and T98G (-0.145) cell lines, it is reasonable to assume that T98G cells depend more strongly on the *NANOG* gene. Despite showing a decrease, no significant difference in *NANOG* expression between our control and CB-HSC-NK treated groups was evident.

Many experts believe that mutations in the *IDH1* and *IDH2* genes play a crucial role in the onset of various types of gliomas. These mutations determine a specific path of oncogenic progression and can result in a more favorable clinical outcome in these cancers. They are commonly found in grade II and grade III oligodendrogliomas and astrocytoma, as well as secondary glioblastoma, but not in primary glioblastoma [60]. Recently, the 2021 WHO Classification of Tumors of the CNS eliminates the term "Glioblastoma, IDH-mutant" with "astrocytoma, IDH-mutant" within grading of II-IV [61]. In a genome-wide study of Parsons, the uncommon IDH1 mutation in glioblastoma is seen in younger patients and associated with increase in overall survival [62]. IDH-mutant gliomas decrease their expression of NK cell activation receptor ligands (NKG2DLs) such as ULBP1, and ULBP3 through hypermethylation [63]. Therefore, escapes NK cells mediated lysis. DepMap data shows that the model glioblastoma T98G cell line used in our study does not possess IDH mutation. This might reinforce the idea of using NK cell immunotherapy against IDH1 wild-type which is more commonly seen in primary glioblastoma. Interestingly, a recent computational analysis by Luoto reported that, IDH1-mutated glioblastoma cell line showed lower expression and higher DNA methylation of MHC-I-type *HLA* genes in effort to evade immune system while the NK cells can target these MHC-I deficient tumor cells [64]. Further investigations are warranted to evaluate the relationship of IDH status and NK cell immunotherapy in glioblastoma. The *CDKN2A (Cyclin Dependent Kinase Inhibitor 2a/Multiple Tumor Suppressor 1)* gene located at the human 9p21.3 locus, also known as the *P16* gene, encodes Multiple Tumor Suppressor 1 (MTS1), which belongs to the INK4 family. Mutation of the *CDKN2A* gene will remove the inhibition of the CyclinD-CDK4 complex. Then, RB protein phosphorylation results in abnormal cell cycle progression, and cells gain unlimited proliferation ability [65,66]. In most IDH wild-type GBM, the cell cycle control of pRB pathway is alternated due to homozygous deletion of *CDKN2A/B*, amplification of *CDK4/6*, and change of *RB1* gene [67]. In a recent study by Han, researchers have analyzed frequency of 9p21 loss in 12 tumor cohort including GBM and their correlation with tumor-immune microenvironment phenotypes; they have shown decrease in abundance of B cells, T cells, NK cells, T follicular helper cells, memory CD4 T cells, and cytotoxic lymphocytes as well as cytokines regulating immune cell recruitment [68]. Thus, it may be feasible to study further investigate the CB-HSCs-derived NK cells interaction with T98G glioblastoma cell line which possess *CDKN2A* deletion and *CDK6* amplification.

Conclusion

In summary, our findings suggest that NK cells can be differentiated from CB-HSCs on the UC- MSC feeder layer successfully with strong expression of highly specific NK cell cytotoxicity receptors. Cytotoxic CB-HSCs-derived NK cells induce apoptosis of glioblastoma cells *in vitro*. Additionally, these NK cells can down regulate *KRAS* oncogene expression. We here provide a potential combined approach to harvest NK cells from a discarded material to develop immunotherapeutic against glioblastoma. To the best of our knowledge, this is the first study that differentiates NK cells from CB-HSCs on the UC-MSC feeder layer and investigates the cytotoxic capability against the T98G glioblastoma cell line. Future strategies to exploit these NK cells as an off-the-shelf cellular product that can be loaded in an intrathecal delivery system such as baclofen pumps may help fight this most aggressive brain cancer.

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Author Contributions

C.E. performed data analysis and wrote the manuscript. O.L. and E.K.H. performed the experiments. G.E. and V.S.H. designed the study, provided critical discussion and comments, revised the manuscript. All the authors reviewed the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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