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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.glioblastoma celA51tTJ/C20 12.655714

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. is aggressive behaviour of this cancer correlates with its poor survival rates. e 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. is 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]. A er 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 *ia* various surface receptors. ese 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]. ey 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 di culties, such as producing su cient, highly puri ed, 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- *i* , enabling manipulations or a er infusion of manufactured NK cells, which yielded more satisfactory results [20-22].

For these reasons, we here focused on obtaining NK cells by di erentiating Cord Blood Hematopoietic Stem Cells (CB-HSC) ex- *i* . Most of the umbilical cord is discarded a er birth, although it possesses a rich source of stem cells. e 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 di erentiated into NK cells a er 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 di erentiation and expansion to mimic their physiological supporting properties during haematopoiesis. ey 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 pro le [25]. While being superior in expansion properties and easier to harvest [26]. UC-MSCs also promote the expansion of HSCs synergistically with cytokines ex *i* [27,28].

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

Ma e ial an Me ho

Cell c l e

UC-MSCs and CB-HSCs were obtained from Erciyes University Genome and Stem Cell Center, Kayseri, Turkey. e cells were thawed and transferred into T25 culture asks at a density of 1×10^3 cell/ml. Fi h 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 modi ed 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 culture9T.a(t)6 ()11 (t)lembtain (e (a)2.37(n)4 (d 100)]TJETEMC /P <</Lang (e1-US)/MCID 20/TT13 (Tf96 B7 B

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3E and 3F respectively). NKG2D and NCR1 are two major activating receptors responsible for NK cell cytotoxicity. e 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 di erentiated. Control staining was negative (Figure 3A).

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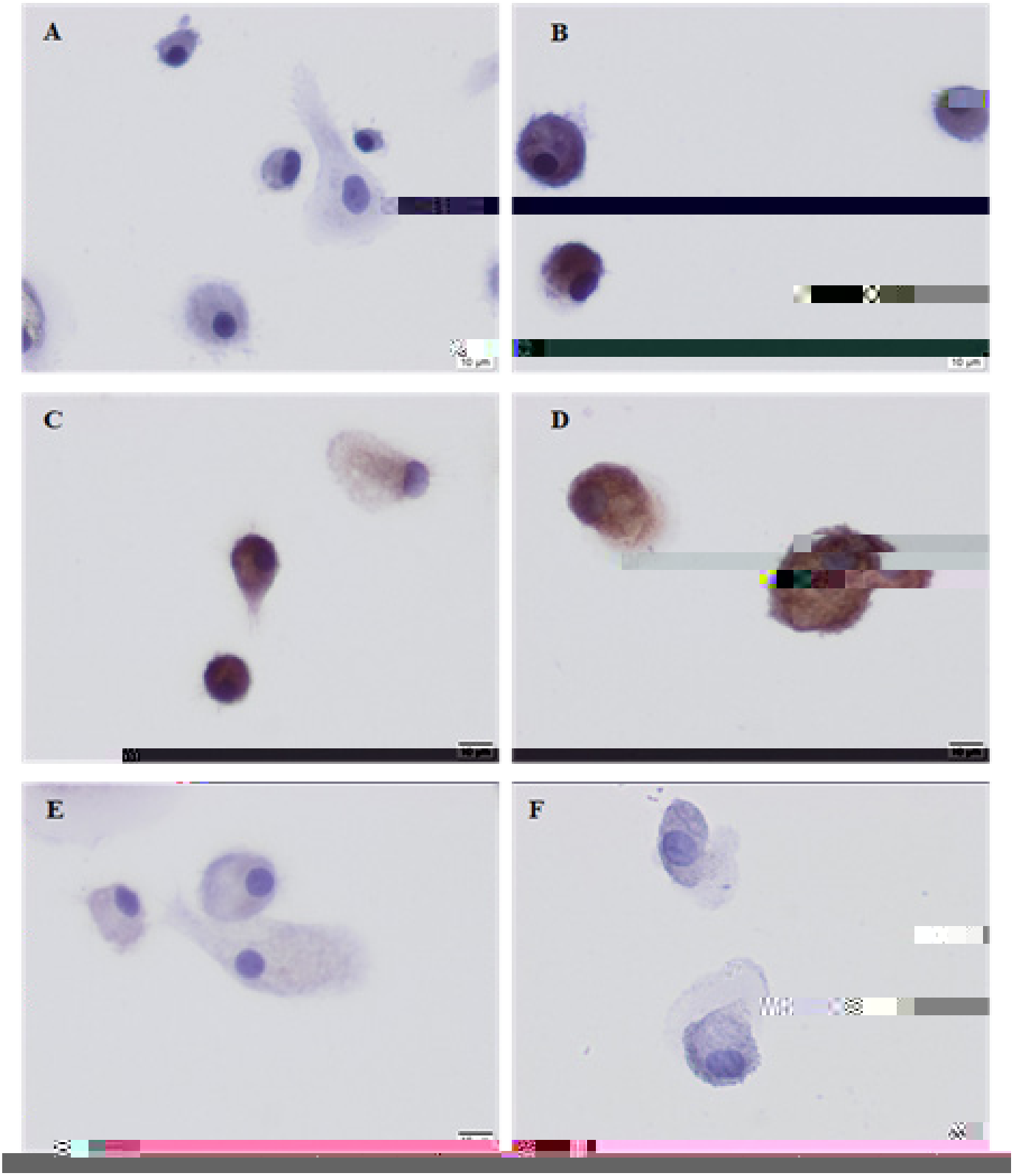

Figure 3: Control (A) *NCAM1*, (B) NKG2D, (C) *NCR1*, (D) *CD34*, (E) CD90, (F) Staining in CB-HSC-NK cells. Scale bars: 10 µm. *NCAM1*: Neural Cell Adhesion Molecule 1; NCR1: Natural Cytotoxicity Triggering Receptor 1; NKG2D: Natural Killer Group 2D; CD: Cluster of Diferentiation fow cytometry results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

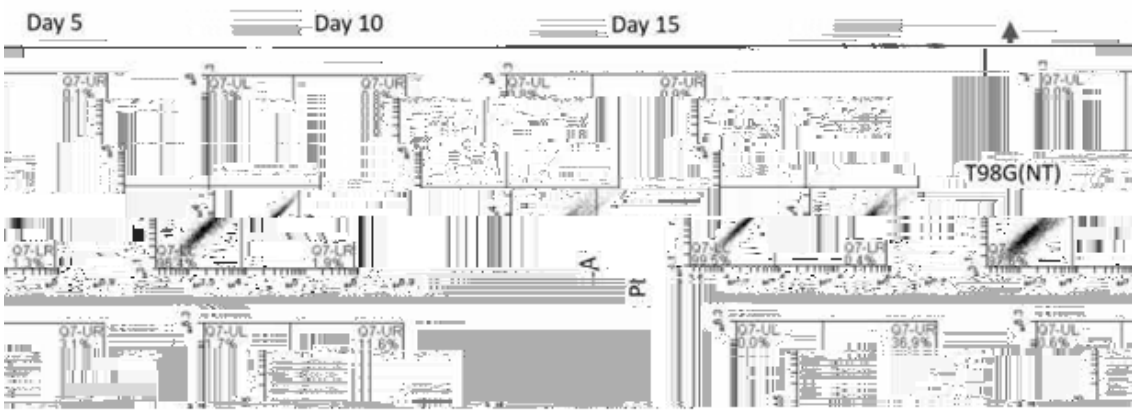

Figure 4: Efect of NK cells diferentiated from cord blood hematopoietic stem cells on the induction of apoptosis of T98G glioblastoma cells.

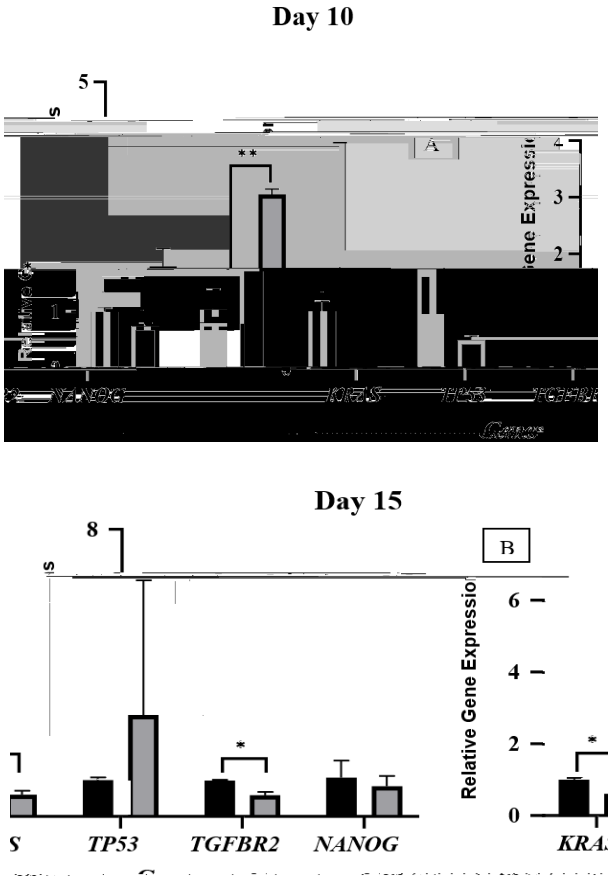

Figure 5: Variations in gene expression of *KRAS*, *TP53*, *TGFBR2* and *NANOG* genes in T98G cells after co-cultured with CB-HSC-NK cells compared to the T98G cells without co-culturing as the control group on (A) Day 10, and (B) Day 15. **Note:** Control; T98G+CB-HSC-NK; **p<0,01, ***p<0,001 T98G+CB-HSC-N..

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

D i c ion

More than 250 000 people are a ected 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 e ects in both *i i* and *i i* 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 e ective, one of the major limiting factors for NK cell therapy is insu cient production. To overcome this obstacle, we tested an alternative method to obtain NK cells by di erentiating Cord Blood-Hematopoietic Stem Cells (CB-HSCs) on umbilical cord-mesenchymal stem cells (UC-MSC) feeder layer. An umbilical cord is o en discarded as a waste material a er 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 di erentiated into cytotoxic NK cells a er ve 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- broblast feeder layer derived from Wharton's jelly of the umbilical cord in the presence of di erent concentrations of cytokines for two weeks. Using this method, they obtained a signi cant 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 di erentiation-inducing and proliferative e ects of the cytokines released from the UC-MSCs in both the early and late stages of NK cell di erentiation from CB-HSCs. We applied a protocol to induce di erentiation 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. e use of mitomycin-C to inhibit the proliferation of UC-MSCs did not result in cell death and maintained viability until the nal day of the experiment. At the end of our protocol, we achieved fully di erentiated NK cells that lack stem cell markers and express activating receptors. Notably, our di erentiated 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 speci c for NK cells [40,41]. NK p46 is also involved in target recognition *ia* still-unidenti ed 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.

A er 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 d on all

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]. e 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 e ect information for each gene in cancer cell lines. By comparing *NANOG* gene e ect 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 signi cant di erence 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. ese mutations determine a speci c path of oncogenic progression and can result in a more favorable clinical outcome in these cancers. ey 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 Classi cation 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]. erefore, escapes NK cells mediated lysis. DepMap data shows that the model glioblastoma T98G cell line used in our study does not possess IDH mutation. is 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 e ort to evade immune system while the NK cells can target these MHC-I de cient tumor cells [64]. Further investigations are warranted to evaluate the relationship of IDH status and NK cell immunotherapy in glioblastoma. e *CDKN2A (C cliy Depeydey Kiya e Iyhibi 2a/M l iple T m S pp e 1)*

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