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Original Article

Bulk RNA sequencing reveals the comprehensive genetic characteristics of human cord blood-derived natural killer cells

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ABSTRACT

Introduction: Innate immune cells are important in tumor immunotherapy. Natural killer cells (NKCs) are also categorized as innate immune cells and can control tumor growth and metastatic spread. Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. NKC-based immunotherapy is a promising treatment strategy against GBM. We previously reported a feeder-free expansion system that yielded large-scale highly purified and cytotoxic NKCs derived from human cord blood (CB). In the present study, we performed comprehensive genomic analyses of NKCs generated from human CB (CBNKCs) as compared those from human peripheral blood (PB) (PBNKCs).

Methods: Frozen T cell-free CB mononuclear cells were cultured with recombinant human interleukin (rhIL)-18 and rhIL-2 in anti-NKp46 and anti-CD16 antibody immobilization settings. After 14-day expansion, the total RNA of the CBNKCs or PBNKCs was extracted and transcriptomic analyses was performed to determine their similarities and differences. We also examined CBNKC and PBNKC activity against a GBM cell line.

Results: Differential expression gene analysis revealed that some NK activating and inhibitory receptors were significantly downregulated in the CBNKCs compared to PBNKCs. Furthermore, genes related to anti-apoptosis and proliferation were upregulated in the CBNKCs. Enrichment analysis determined that the gene sets related to immune response and cytokines were enriched in the CBNKCs. Gene set enrichment analysis demonstrated that the immune response pathway was upregulated in the CBNKCs. Cytotoxic assays using impedance-based cell analyzer revealed that the CBNKCs enhanced NKC-mediated cytotoxicity on GBM cells as compared to the PBNKCs.

Conclusions: We demonstrated the characteristics of human CBNKCs. Cell-based therapy using the CBNKCs is promising for treating GBM.

Abbreviations: CAR, chimeric antigen receptor; CB, cord blood; CBMCs, cord blood mononuclear cells; CBNKCs, cord blood-derived natural killer cells; DEGs, differentially expressed genes; FBS, fetal bovine serum; GBM, glioblastoma; GO, gene ontology; GSEA, gene set enrichment analysis; GVHD, graft-versus-host disease; IFN-γ, interferon gamma; MACS, magnetic-activated cell sorting; NKCs, natural killer cells; PB, peripheral blood; PBNKCs, peripheral blood-derived natural killer cells; PBS, phosphate-buffered saline; rhIL-18, recombinant human IL-18; RNAseq, RNA sequencing; RTCA DP, real-time cell analysis dual-purpose; TPM, transcripts per million.

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1. Introduction

Natural killer cells (NKCs) were discovered more than 40 years ago and are key innate immunity elements that can mediate anti-tumor and antiviral responses to kill cancer cells or infected cells. NKCs have recently attracted attention for their potential in cell-based immunotherapies. NKCs can recognize cancer cells based on a balance between activating and inhibitory receptors without antigen sensitization [1,2]. While cytolytic T lymphocytes recognize tumor-derived peptides with MHC class I molecules, NKCs also sense and are activated by the lack of MHC molecules [3]. Furthermore, NKCs can eliminate tumors that downregulate MHC class I expression [4]. NKC-mediated cytotoxicity and cytokine release can affect the activity of other innate immune cells, such as dendritic cells and macrophages, and neutrophils [5,6]. Furthermore, NKC-based immunotherapy potentially drives the cancer immunity cycle and improves patient outcome. These NKC properties are important in the development of therapeutic strategies in cancer.

While T cell-based immunotherapy often induces graft-versus-host disease (GVHD), NKC-based immunotherapies do not due to their lack of T-cell receptors [7,8]. When tumor cells lack well-defined antigens for a specific T cell response, NKCs present the possibility of the use of allogeneic products and killing such tumors. These features enable NKC administration in multiple patients without causing GVHD [7,8]. Recently, a clinical trial demonstrated that ex vivo expanded allogeneic NKCs exhibited enhanced anti-tumor activity against myeloid leukemia. Five of nine evaluable patients presented good response, which included four complete remissions with low toxicity [9]. Furthermore, NKCs do not induce GVHD, cytokine release syndrome, or immune effector cell-associated neurotoxicity syndrome, which demonstrates their low toxicity [10]. Considering the immunosuppressed condition in patients with cancer, allogeneic NKC-based immunotherapy potentially leads to an effective anti-tumor effect with less toxicity compared to T cell-based immunotherapy.

Primary NKCs can be derived from peripheral blood (PB) or umbilical cord blood (CB) [11]. Whereas PB-derived NKCs (PBNKCs) generally require apheresis from healthy donors, CB-derived NKCs (CBNKCs) are available frozen off-the-shelf through blood banks. The frequency of NKCs in PB is 10–15% of lymphocytes, but is ~30% in CB [12]. As there are more than 600,000 banked CB units worldwide [13], they represent a unique opportunity as an available donor source to utilize CB for NKC-based immunotherapy. Accordingly, CBNKCs are promising effector cells for cancer immunotherapy.

Previously, we reported a feeder-free expansion system that yielded large-scale highly purified and cytotoxic human CBNKCs using a combination of cytokines and antibodies targeting NK receptors [14–16]. In the present study, we evaluated the gene expression profiles of CBNKCs as compared to PBNKCs using RNA sequence analysis and anti-tumor activity against a GBM cell line.

2. Materials and methods

2.1. Human CB mononuclear cells (CBMCs)

CBMCs were acquired from the RIKEN BioResource Research Center (RIKEN BRC; Tsukuba, Ibaraki, Japan) following approval of the Nara Medical University Ethics Committee (approval number 3310) according to the applicable guidelines.

2.2. Antibody-coated plates

Anti-human NKp46 antibodies (clone 195, 314, R&D Systems, Minneapolis, MN, USA) and anti-human CD16 antibodies (clone 3G8, Thermo Fisher Scientific, Waltham, MA, USA) (both, 5 mg/mL) were prepared in phosphate-buffered saline (PBS; Kohjin Bio, Saitama, Japan) containing 0.1% human serum albumin (FUJIFILM Wako Pure Chemical, Tokyo, Japan). The antibody solution (1.5 or 0.7 mL) was transferred to 24- or 12-well plates (Corning, Steuben, NY, USA) and incubated at 4 °C for >12 h for antibody immobilization. The antibody solution was then removed from the flask, the flask was washed with PBS, and subsequently used for human NKC culturing.

2.3. NKC expansion

The human PBNKC and CBNKC expansions were conducted as described previously [14]. Briefly, the frozen CBMCs from RIKEN BRC were obtained from three volunteers (0 years old, one man and two women). The CD3 fraction was depleted by magnetic-activated cell sorting (MACS) using Mini MACS columns and CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The CBMCs were incubated with the CD3 MicroBeads and $CD3^+$ cells were depleted using an LD column (Miltenyi Biotec). For the PBNKC expansion, peripheral blood mononuclear cells (PBMCs) were obtained from 16 mL heparinized peripheral blood from three healthy mal volunteers (51, 47, and 43 years old). The PBMC CD3 fraction was delpleted using RosetteSep™ Human CD3 Deplettion Cocktail (STEMCELL Technologies, Vancouver, Canada). The CD3-depleted cells (2×10^6 or 1×10^6) were plated in 6- or 12-well anti-NKp46 and anti-CD16 antibody immobilization plates containing AIM-V medium (Thermo Fisher Scientific) supplemented with 10% AB plasma from the healthy donor or 10% autologous plasma, 50 ng/mL recombinant human IL-18 (rhIL-18, Medical & Biological Laboratories, Nagoya, Japan), and 3000 IU/mL rhIL-2 (COREFRONT, Tokyo, Japan) at 37 °C in a humidified incubator containing 5% CO_2. When the color of the medium turned yellow, indicating that the cell number had reached confluence, a double volume of AIM-V medium supplemented with only 3000 IU/mL of rhIL-2 was added. The expansion culture was performed until 14 days. The expanded cells were frozen in CELLBANKER 2 (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan). We have already reported the similarity of the NK cells purity between CBNKCs and PBNKCs [14,15].

To assess the inhibitory effects on tumor cell growth, the frozen cells were revived in AIM-V medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; MP Biomedicals, Tokyo, Japan) and 3000 IU/mL rIL-2, followed by a 2-day incubation.

2.4. Human glioblastoma (GBM) cell line

This study used a standard T98G human GBM cell line (RIKEN BRC) known for its sensitivity to NKC activity [16]. The cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) enriched with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Thermo Fisher Scientific) at 37 °C in a humidified environment containing 5% CO_2.

2.5. Cytotoxic assays

The inhibitory effects of the NKCs on the T98G cells were investigated using xCELLigence RTCA DP (real-time cell analysis dual-purpose) instruments (ACEA Biosciences, San Diego, CA, USA) using a previously described procedure [14,15,17,18]. Briefly, complete medium (100 μL) was added to each well on E-plate 16 (ACEA Biosciences), and background impedance was measured at 37 °C in a humidified atmosphere containing 5% CO_2. T98G cells [2×10^4 per well (50 μL)] were seeded in each well as the target (T) cells for 20 h. The expanded NKC-containing populations (50 μL) were

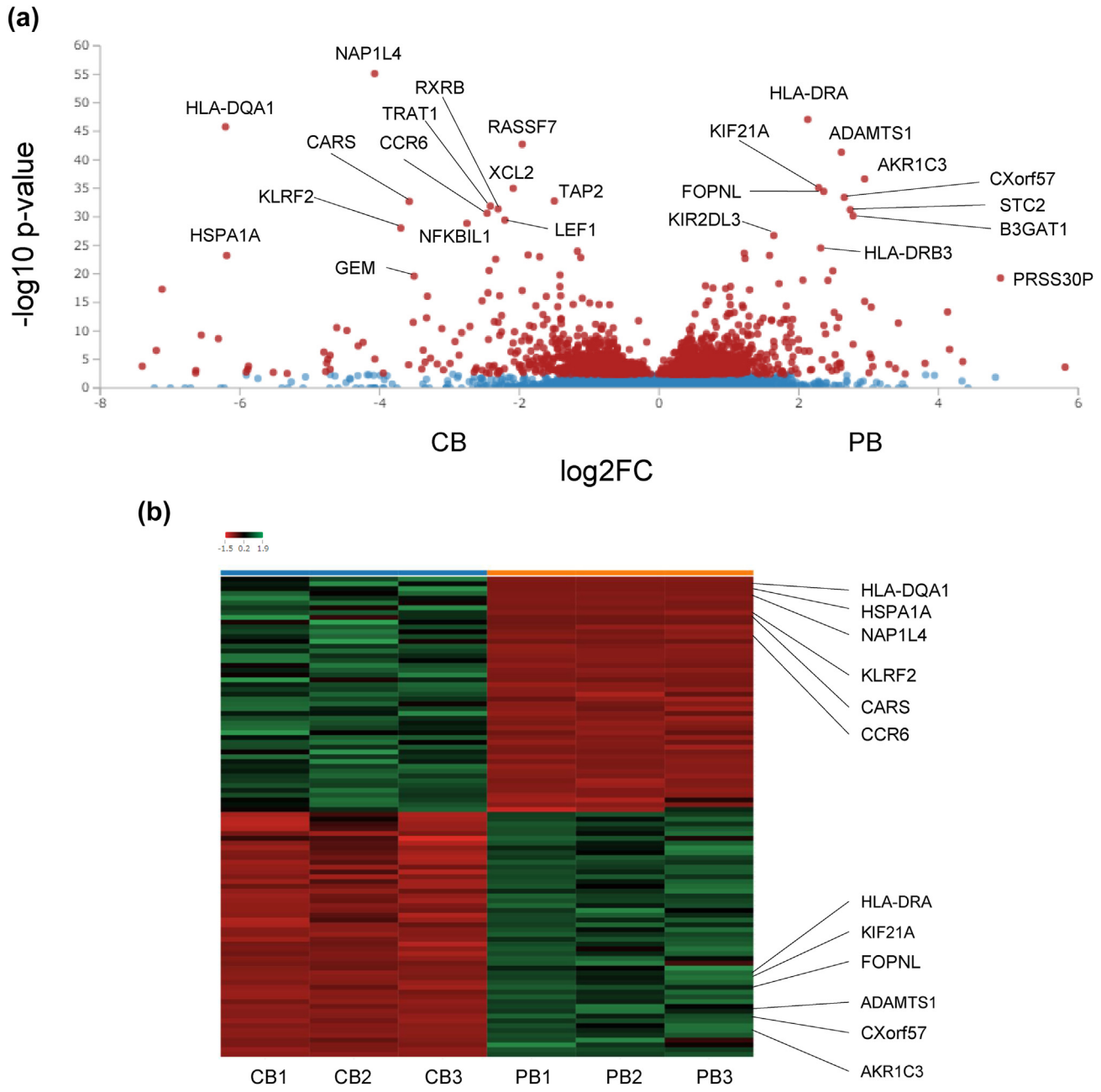


Fig. 1. DEG and enrichment analyses. a. Volcano plot of differential expression analysis results shows the log2 scaled fold change (x-axis) and the minus log10 p-value (y axis) of each gene. Red dots indicate the genes with a significant expression change. b. Heatmap of expression values (normalized as TPMs) of selected genes in each sample.

added to each well as effector (E) cells at E:T ratios of 0.5:1. The impedance measurement was recorded every 5 min for 6 h. The data were analyzed using RTCA Software Package 1.2 (ACEA Biosciences). Cytotoxicity was calculated from impedance values (cell index) using a previously reported method [19] with slight modifications. Cytotoxicity (%) was calculated using the following formula: (1 − normalized cell index of target cells co-cultured with each sample ÷ normalized cell index of target cells) × 100.

2.6. RNA sequencing (RNAseq)

The total RNA of CBNKCs and PBNKCs cultured for 14 days was extracted using NucleoSpin RNA (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The total RNA underwent next-generation sequencing at Amelieff (Tokyo, Japan). The sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA, Vat No. 7760) following the manufacturer's protocol. The resulting libraries were purified by the AMPure XP system (Beckman Coulter, CA, USA) and library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent, CA, USA). Sequencing was performed with NovaSeq 6000 (Illumina, CA, USA) with a 150-base pair-end read setting. Reads were aligned to the *Homo sapiens* reference genome (hg38) using RNA STAR. The read counts per gene were obtained using featureCounts (version 2.0.0). The relative expression level of each gene was normalized by the transcripts per million (TPM). Furthermore, the genetic characteristics were analyzed and visualized with RNAseq [20].

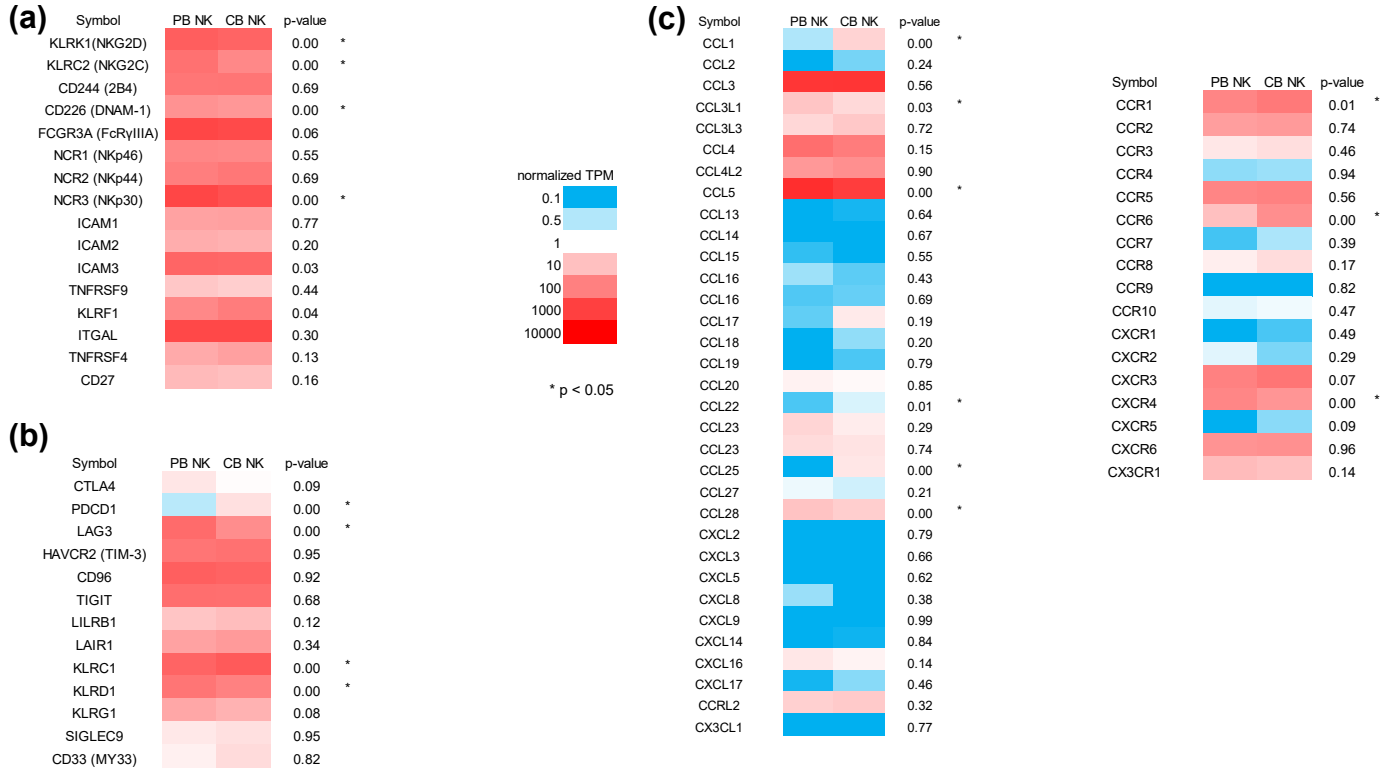


Fig. 2. Heatmap of expression values (normalized as TPMs) of selected NKC-related genes in each sample. a. NK activating receptor. b. NK inhibitory receptor. c. Chemokines and chemokine receptor. d: Cytotoxicity. e. Immunosuppression. f. Anti-apoptosis. g. Inflammatory cytokines. h: Proliferation. i. GO analysis of CBNKC enriched gene set. Statistical differences were determined by Student's *t*-test. *$p < 0.05$.

2.7. Statistical analysis

Statistical analyses were conducted with Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The statistical significance of differences was determined using Student's *t*-test. $P < 0.05$ was deemed statistically significant.

3. Results

3.1. RNAseq analysis of CBNKCs and PBNKCs

Transcriptome profiling of CBNKCs versus PBNKCs was performed to identify differentially expressed genes (DEGs). Bulk RNA sequences were used with three experimental replicates each. The CBNKCs contained a total of 143 significantly upregulated genes and 100 significantly downregulated genes (Fig. 1a and b). We also assessed the expression of NKC immunity-related genes: activating receptors (*KLRK1*, *KLRC2*, *CD244*, *CD226*, *FCGR3A*, *NCR1*, *NCR2*, *NCR3*, *ICAM1*, *ICAM2*, *ICAM3*, *TNFRSF9*, *KLRF1*, *ITGAL*, *TNFRSF4*), inhibitory receptors (*CTLA4*, *PDCD1*, *LAG3*, *HAVCR2*, *CD96*, *TIGIT*, *LILRB1*, *LAIR1*, *KLRC1*, *KLRD1*, *KLRG1*, *SIGLEC9*, *CD33*), chemokines (*CCL1–4*, *CCL4L2*, *CCL5*, *CCL13–23*, *CCL25*, *CCL27*, *CCL28*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL8*, *CXCL9*, *CXCL14*, *CXCL16*, *CXCL17*, *CCRL2*, *CX3CL1*), chemokine receptors (*CCR1–10*, *CXCR1–6*, *CX3CR1*), cytotoxicity (*GZMA*, *GZMB*, *GZMH*, *GZMM*, *GZMK*, *CXCR1*, *FASLG*, *PRF1*, *TNFSF10*), inflammatory cytokines (*IFNG*, *IL1B*, *IL1A*, *IL7*, *IL12A*, *IL15*, *TNF*, *CSF2*), immunosuppression (*IL10*, *IL13*, *IL23A*, *TGFB1*, *TGFBR2*, *TGFBR3*), anti-apoptosis (*BAK1*, *BAX*, *BCL2*, *BCL2L1*, *BCL2L11*, *BID*, *BMF*, *MCL1*), and proliferation (*AURKA*, *FOXM1*, *MKI67*, *PCNA*, *PLK1*). We selected these genes from the our study which is previously published [21] (Supplementary table 1).

Among the CBNKC NKC activating receptors, *KLRC2* (NKG2C), *KLRK1* (NKG2D) *CD226* (DNAM-1), and *NCR3* (NKp30) were significantly downregulated (Fig. 2a). Among the CBNKC NKC inhibitory receptors, *LAG3* and *KLRD1* were downregulated, while *KLRC1* and *PDCD1* were upregulated in CBNKCs (Fig. 2b). Among the CBNKC chemokines, *CCL1*, *CCL22*, and *CCL25* were upregulated, and *CCL3L1*, *CCL5*, *CCL27*, and *CCL28* were downregulated. Among the CBNKC chemokine receptors, *CCR1* and *CCR6* were upregulated and *CXCR4* was downregulated (Fig. 2c). Among the CBNKC cytotoxicity-related genes, *PRF1* was upregulated, and *GZMA* and *GZMK* were downregulated (Fig. 2d). Among the CBNKC immunosuppression genes, *TGFBR3* was upregulated (Fig. 2e). Among the CBNKC anti-apoptosis genes, *BCL2L11* was upregulated and *BAX* and *MCL1* were downregulated (Fig. 2f). Among the CBNKC inflammatory cytokines, TNF was upregulated, while no proliferation genes were significantly upregulated or downregulated (Fig. 2g and h).

Gene ontology (GO) analysis revealed that the following CBNKC immune-related gene sets were significantly enriched: regulation of immune response, immune response, interferon gamma (IFN-γ)-mediated signaling pathway, positive regulation of cytokine-mediated signaling pathway, IFN-γ secretion, adaptive immune response, regulation of cytokine production, positive regulation of T-helper 1 cell cytokine production, leukocyte cell–cell adhesion, immune response-activating cell surface receptor signaling pathway, lymphocyte chemotaxis, cytokine secretion involved in immune response, and positive regulation of cytokine production involved in inflammatory response (Fig. 2i).

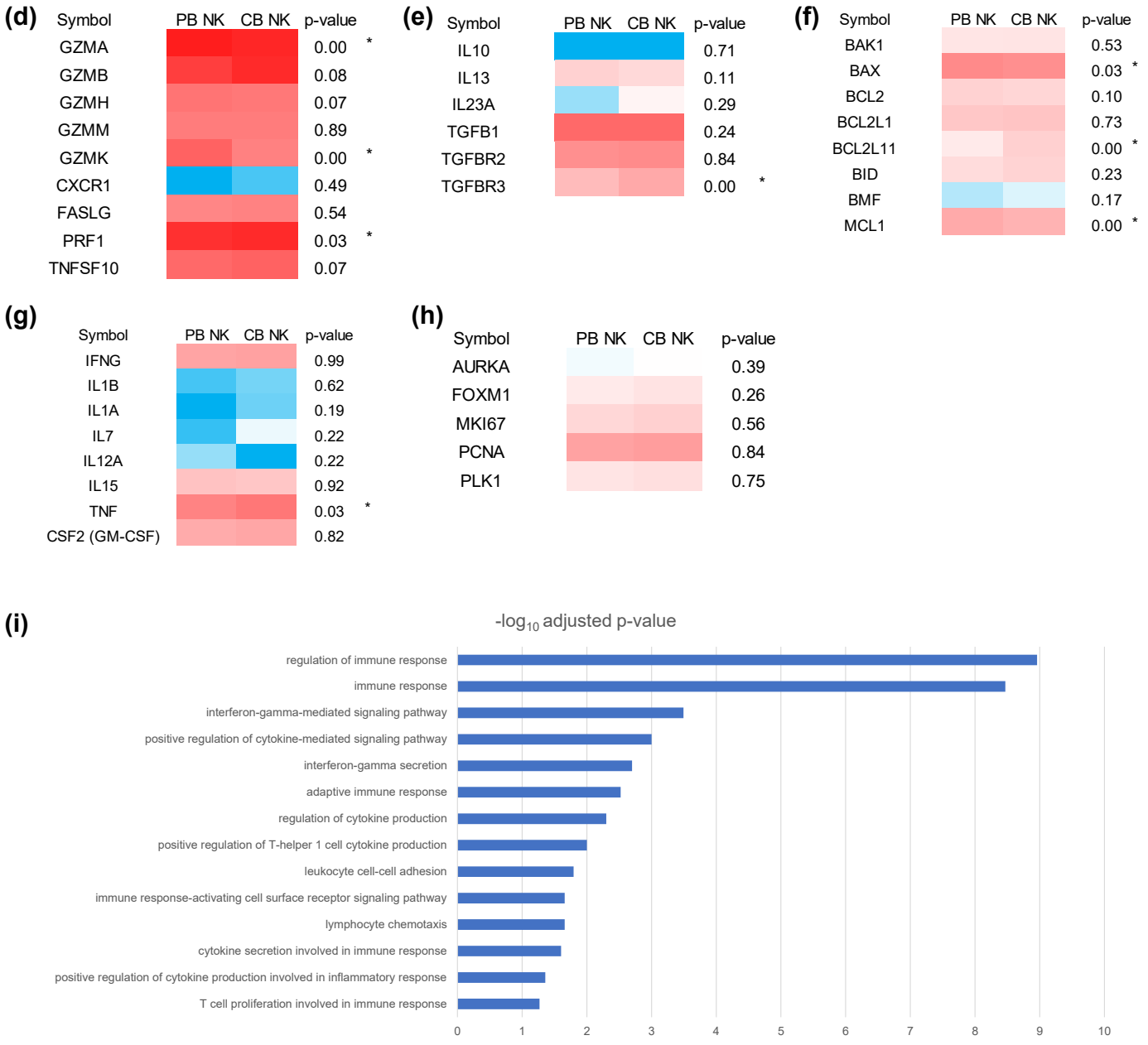


Fig. 2. (*continued*).

All differential expression data we tested was listed in supplementary table 2.

3.2. Gene set enrichment analysis (GSEA) of CBNKCs and PBNKCs

GSEA revealed that the following CBNKC pathways were significantly upregulated: proteasome degradation, transport of mature mRNA derived from an intron-containing transcript, ribosome biogenesis in eukaryotes, transport of mature transcript to cytoplasm, processing of capped intron-containing pre-mRNA, proteasome, tRNA processing, splicesome, transport of the SLBP-dependent mature mRNA, and export of viral ribonucleoproteins from nucleus (Fig. 3a and c). The following CBNKC pathways were significantly downregulated: eukaryotic translation elongation, cytoplasmic ribosomal proteins, peptide chain elongation, selenocysteine synthesis, ribosome eukaryotes, viral mRNA translation, formation of a pool of free 40S subunits, SRP-dependent co-translational protein targeting to membrane, and eukaryotic translation termination (Fig. 3b and d). The significantly enriched CBNKC immune-related pathways were antigen presentation: folding, assembly, and peptide loading of class I MHC, and immune response (Fig. 3e and f).

3.3. Cytotoxic assay

The growth inhibition effects of CBNKCs and PBNKCs on the T98G GBM cell line were investigated using an RTCA system. The T98G cells were seeded and cultured for 1 day, then CBNKCs or PBNKCs were added to each well at a 0.5:1 E:T cell ratio. Compared to the PBNKCs, the CBNKCs significantly inhibited T98G cell growth (Fig. 3g).

(a)

Pathway	NES	-log$_{10}$ adjusted p-value
WikiPathways; Proteasome Degradation	2.214	2.09691
REACTOME; Transport of Mature mRNA derived from an Intron-Containing Transcript	2.183	2.09691
KEGG; Ribosome biogenesis in eukaryotes	2.161	2.09691
REACTOME; Transport of Mature Transcript to Cytoplasm	2.153	2.09691
REACTOME; Processing of Capped Intron-Containing Pre-mRNA	2.099	2.09691
KEGG; Proteasome	2.073	2.09691
REACTOME; tRNA processing	2.073	2.09691
KEGG; Spliceosome	2.053	2.09691
REACTOME; Transport of the SLBP Dependant Mature mRNA	2.044	2.09691
REACTOME; Export of Viral Ribonucleoproteins from Nucleus	2.039	2.09691

(b)

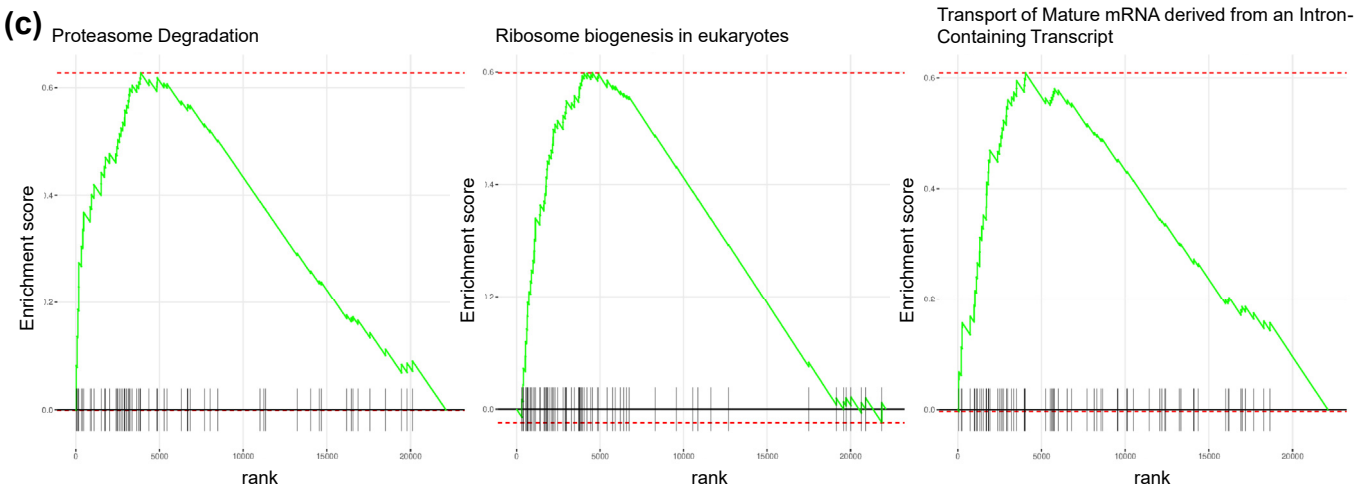
Pathway	NES	-log$_{10}$ adjusted p-value
REACTOME; Eukaryotic Translation Elongation	-3.232	2.09691
WikiPathways; Cytoplasmic Ribosomal Proteins	-3.201	2.09691
REACTOME; Peptide chain elongation	-3.195	2.09691
REACTOME; Selenocysteine synthesis	-3.178	2.09691
KEGG; Ribosome, eukaryotes	-3.16	2.09691
REACTOME; Viral mRNA Translation	-3.129	2.09691
REACTOME; Formation of a pool of free 40S subunits	-3.125	2.09691
REACTOME; SRP-dependent cotranslational protein targeting to membrane	-3.118	2.09691
REACTOME; Eukaryotic Translation Termination	-3.108	2.09691

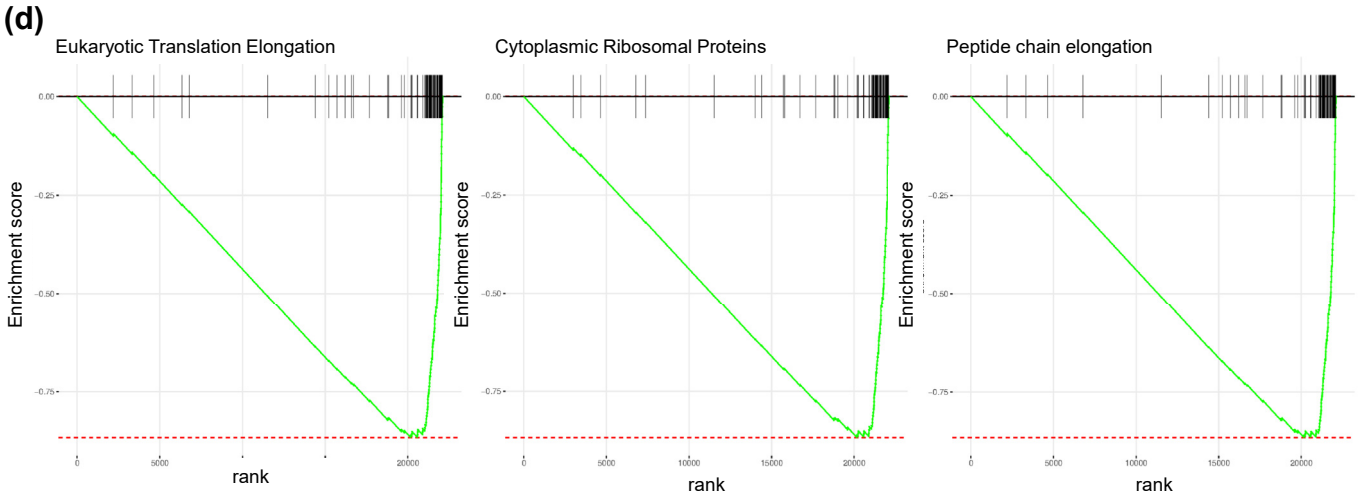
Fig. 3. Transcriptome analysis of CBNKCs by RNAseq and growth inhibition assay against GBM cells. a, b. GSEA revealed the upregulated (a) and downregulated (b) pathways in CBNKCs. c, d. Enrichment plot depicting the upregulated (c) and downregulated (d) pathways. e, f. Upregulated immune-related pathways (e) and enrichment plot (f). g. Bar graphs illustrate the cytotoxicity of 4 h (E:T ratio = 0.5:1). Data are the mean ± SD of two independent experiments ($n = 3$–4). Statistical differences were determined by Student's *t*-test. ***$p < 0.001$.

4. Discussion

Adoptive immunotherapy has been utilized for treating several cancers [22–24], but effective immunotherapy has not been reported in GBM treatment. We focused on NKC-based immunotherapy as NKCs have more advantages in recognizing tumor cells via multiple activating and inhibitory receptors despite the diminished expression of MHC class I molecules and activating cancer immunity cycle compared to T cell-based immunotherapy [5,25,26]. NKCs can be derived from multiple platforms, including PB, umbilical CB, cell line, induced pluripotent stem cells, and embryonic stem cells [16,27–31]. NKCs account for 10% of all lymphocytes in PB, and for up to 30% of the lymphocytes in CB [12]. Unlike PB, CB can be collected and frozen, and easily utilized for adoptive cell immunotherapy [32]. While some platforms expanded CB-derived NKCs, we previously reported a feeder-free expansion system that yielded large-scale highly purified and cytotoxic human CB-derived NKCs [14,33,34].

CBNKCs produce similar amounts of IFN-γ and TNF to PBNKCs, although CBNKCs had weaker cytotoxicity than PBNKCs against K562 cells [27,28]. In the present study, we examined the cytotoxic effect of CBNKCs and PBNKCs against a GBM cell line, and determined that CBNKCs had stronger cytotoxic activity than PBNKCs. This finding indicated that our platform is effective and our





(e)

Pathway	NES	-log$_{10}$ adjusted p-value
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	2.01	2.09691

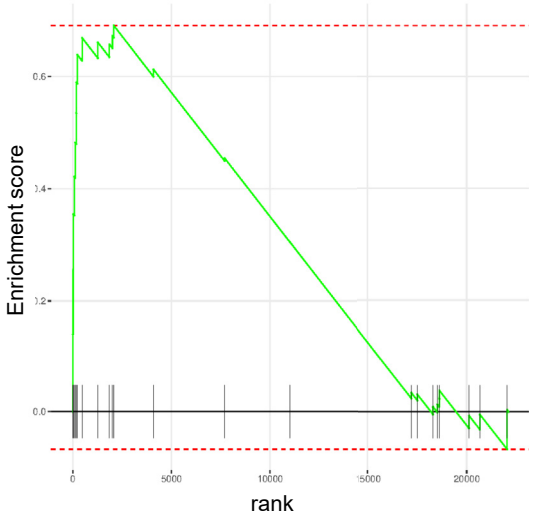
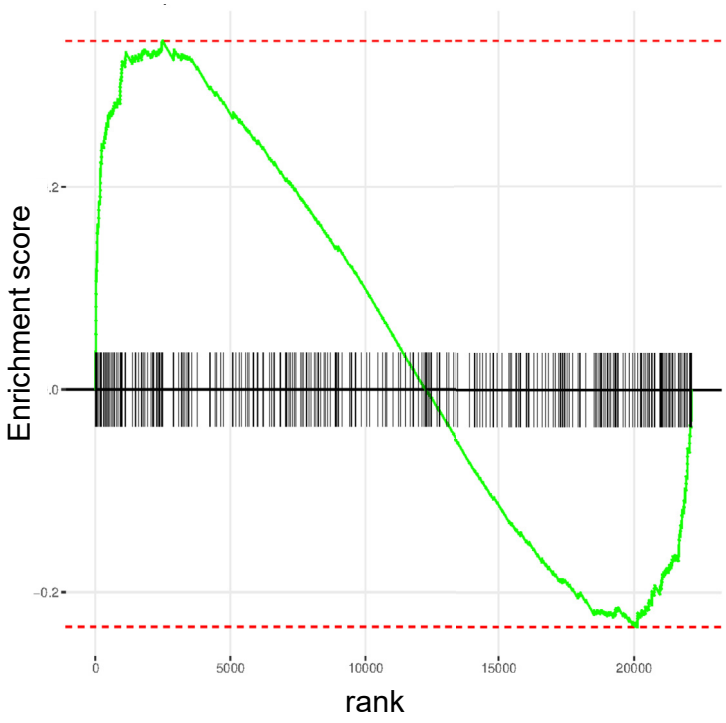


Fig. 3. (*continued*).

(f)

GO_term	NES	-log$_{10}$ adjusted p-value
immune response	1.531	1.769551



(g)

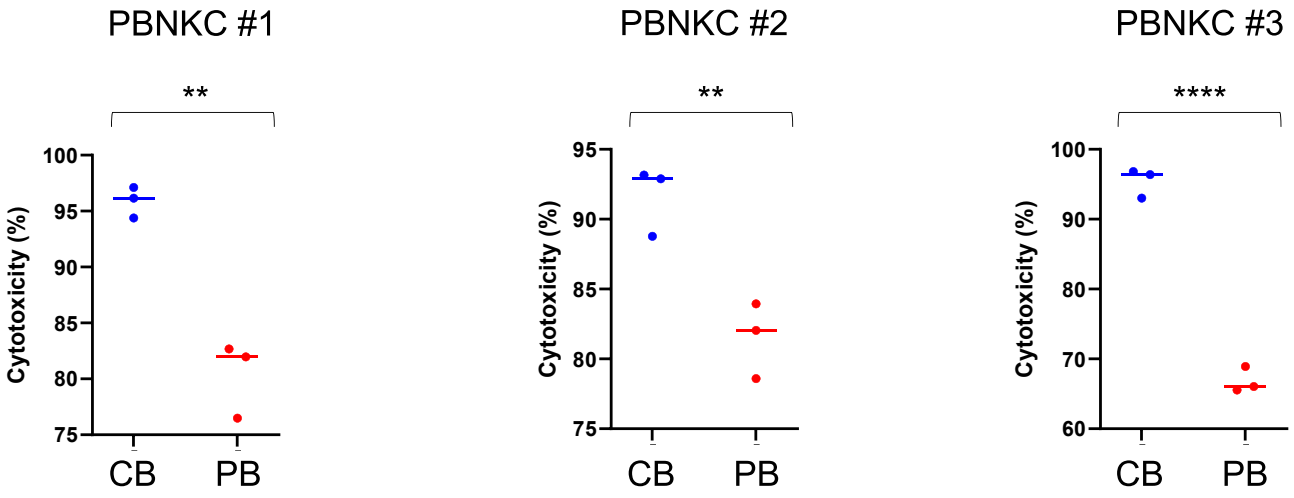


Fig. 3. (*continued*).

established CBNKCs are potential promising agents for GBM treatment.

We also comprehensively explored the DEGs between the CBNKCs and PBNKCs. Generally, the CBNKCs exhibited decreased expression of adhesion molecules and activating receptors, such as *CD2* and *CD16*, and increased expression of the inhibitory receptor *NKG2A* as compared to the PBNKCs [27,28,35]. Furthermore, some NKC activating receptors (NKG2C, DNAM-1, and NKp30) and NKC inhibitory receptors (LAG3 and KLRD1) were downregulated in CBNKCs. The downregulated NKC inhibitory receptors may contribute to the CBNKC anti-tumor activity. Dalle et al. reported that CBNKCs are younger and have stronger proliferation potential than the PBNKCs [27]. Our expression analysis determined that the *BCL2L11* anti-apoptosis gene was upregulated in the CBNKCs.

Additionally, the enrichment analysis demonstrated enriched immune-related gene sets in the CBNKCs, which supported these findings.

GSEA revealed several upregulated pathways in the CBNKCs as compared to the PBNKCs. According to the high normalized enrichment score, the top altered pathways were proteasome degradation, transport of mature mRNA derived from an intron-containing transcript, and ribosome biogenesis in eukaryotes. Proteasome degradation is essential for maintaining cellular homeostasis and regulating various cellular functions [36]. Transport of mature mRNA derived from a intron-containing transcript is a participant of RNA metabolism, which includes capping, slicing, and 3′-cleavage and polyadenylation to yield mature mRNA molecules that are exported from the nucleus [37]. Ribosome biogenesis in eukaryotes is a fundamental cellular process that involves the assembly and maturation of ribosomes, which are essential cellular structures responsible for protein synthesis [38].

In the immune response-related gene sets, immune response and antigen presentation: folding, assembly, and peptide loading of MHC class I were enriched in the CBNKCs. Immune response is a GO term and refers to any immune system process that functions in the calibrated response of an organism to a potential internal or invasive threat. Antigen presentation: folding, assembly and peptide loading of MHC class I is a reactome pathway and is downstream of the immune system [39]. Compared to PBNKCs, all of these upregulated gene sets supported the enhanced anti-tumor activity of CBNKCs against the T98G GBM cell line.

Due to the low risk of GVHD, autologous NKC infusions are the major focus of adoptive NKC-based immunotherapy. However, patients with cancer have systemic immunosuppression status; therefore, they would not yield sufficient amounts of NKCs to treat the cancer. Conversely, allogeneic NKC therapy yields a sufficient number of NKCs with low risk of GVHD. Accordingly, it would be easier to utilize off-the-shelf sources of NKCs, especially CBNKCs. Some studies reported that KIR ligand-mismatched allogeneic NKC treatment induced GVHD in approximately 7% of patients, but this was induced by mixed T cells [40,41].

In summary, we characterized CB-derived NKCs using DEG and enrichment analyses. The gene expression analysis focused on NKC-related genes revealed that the CBNKCs had some upregulated NK activating receptors and downregulated NK inhibitory receptors. Some immune-related gene sets were enriched, and proliferation-related genes were upregulated in the CBNKCs. The CBNKCs also had enhanced anti-tumor activity against GBM cells. Therefore, human CB would be an attractive and promising resource of NKC-based immunotherapy against cancer, including GBM.

Ethics statement

CBMCs were acquired from RIKEN BRC with Nara Medical University Ethics Committee approval (approval number 3310) and following its guidelines.

Author contributions

TN: Conceptualization, design, guidance, methodology, investigation, data analysis, and writing (original draft preparation, review, and editing). TM: Conceptualization, data analysis, and writing (original draft preparation, review, and editing). R. Maeoka, R. Matsuda, TT: Conceptualization and writing (review and editing). FN, MN, SY, YS, IN, Y-SP, HN: Writing (review, and editing). All authors made important contributions to the experiments. All authors have read and agreed to the published version of the manuscript.

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Data availability

All transcriptomics data is deposited Sequence Read Archive (SRA), accession number PRJNA1029346. A link to SRA dataset will also be available at https://www.ncbi.nlm.nih.gov/sra.

Declaration of competing interest

TM is registered with Nara Medical University as a postdoctoral fellow member paying registration fees.

Acknowledgments

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.02.002.

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