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An Extended Flow Cytometry Evaluation of ex Vivo Expanded NK Cells Using K562.Clone1, a Feeder Cell Line Manufactured in Brazil

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A B S T R A C T

Natural killer (NK) cells play a crucial role in the immune system's response against cancer. However, the challenge of obtaining the required quantity of NK cells for effective therapeutic response necessitates the development of strategies for their ex vivo expansion. This study aimed to develop a novel feeder cell line, K562. Clone1, capable of promoting the ex vivo expansion of NK cells while preserving their cytotoxic potential. he K562 leukemic cell line was transduced with mbIL-21 and 4-1BBL proteins to generate K562.Clone1 cells. NK cells were then co-cultured with these feeder cells, and their expansion rate was monitored over 14 days. The cytotoxic potential of the expanded NK cells was evaluated against acute myeloid leukemia blasts and tumor cell lines of leukemia and glial origin. Statistical analysis was performed to determine the significance of the results. The K562.Clone1 co-cultured with peripheral NK showed a significant increase in cell count, with an approximate 94-fold expansion over 14 days. Expanded NK cells demonstrated cytotoxicity against the tested tumor cell lines, indicating preservation of their cytotoxic characteristics. Additionally, the CD56, CD16, inhibitory KIRs, and activation receptors were conserved and present in a well-balanced manner. The study successfully developed a feeder cell line, K562.Clone1, that effectively promotes the expansion of NK cells ex vivo while maintaining their cytotoxic potential. This

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development could significantly contribute to the advancement of NK cell therapy, especially in Brazil.

INTRODUCTION

Since the inception of cell therapy in 1957, marked by the pioneering allogeneic transplantation performed by E. Donnall Thomas, there has been continuous development of advanced therapies for the treatment of cancer, infectious diseases, and genetic disorders [1]. Over time, cell therapy has undergone significant advancem ents and has gained global recognition as the standard procedure for various types of leukemia. Bone marrow transplantation is now routinely conducted in numerous specialized centers worldwide, benefiting from the widespread availability of protocols, supplies, knowledge, and enhanced connectivity [2,3].

Notably, cell therapy has revolutionized treatment approaches, and the integration of immune cells in bone marrow transplantation protocols has shown promise in enhancing the graft-versus-host response, thereby facilitating long-term effectiveness against leukemia [2,3]. Immune cell therapy, also known as immunotherapy, is a treatment approach that aims to enhance the efficiency and effectiveness of the immune system in targeting cancer cells. This can be achieved by stimulating the immune system or providing it with additional components, such as engineered cells, to bolster its response against cancer [4].

In 2017, the Food and Drug Administration approved the first chimeric antigen receptor (CAR) T cell-based therapies (CAR-T) for the treatment of acute lymphoblastic leukemia (ALL) in children and advanced lymphoma in adults [4]. Since then, there has been a significant increase in the number of clinical trials involving CAR-T, with 1087 trials registered on ClinicalTrials.gov as of January 25, 2023. These therapies represent a significant advancement in the treatment of hematologic cancers, including leukemia and multiple myeloma [5].

CAR-T has demonstrated significant promise in the treatment of cancers. Ongoing research in this field is primarily focused on improving the efficacy of CAR-T. One notable challenge is the occurrence of severe off-target side effects and toxicity, which can result from cytokine release syndrome (CRS) following the infusion of modified T cells. Additionally, the development of tumor resistance to CAR T cells is a common obstacle that can lead to relapse and reduce the therapy's effectiveness [4].

Natural killer (NK) cells are considered a safe alternative to T cells in terms of accessibility and safety. Compared to CAR-T, CAR-NK cell therapy has been found to have more manageable cytotoxicity and adverse events. Additionally, NK cells carry a lower incidence of graft-versus-host disease (GVHD), making CAR-NK a promising immunotherapy option for allogenic cell transplantation [6].

NK cells exhibit specific antitumor effects and are associated with fewer off-target complications, due to their ability to recognize major histocompatibility complex I (MHC-I) expressed on healthy cells and to avoid attacking them. This singular mechanism for distinguishing between tumor and healthy cells, based on the missing-self theory, is a balance between NK group-2 receptors (NKG2 type) and killer cell immunoglobulin-like receptors (KIRs) [7,8].

Challenges in the field of NK cell therapy include determining the best source of NK cells and developing an effective in vitro expansion strategy [6]. The NK cells can be sourced from peripheral blood, in which they typically constitute between 5% and 15% of the total lymphocyte population [9]. Another potential source of NK cells is umbilical cord blood, which typically exhibits greater NK cell expression at approximately 30% of the total lymphocyte population [10]. However, cord blood does not provide the ideal number of cells required to achieve a therapeutic response, which is estimated to be between 1×10^5 and 1×10^9 NK cells per kilogram of patient body weight [11-13].

NK cells can be expanded through activation with proliferative cytokines, such as IL-2 and IL-15. However, the use of feeder cells, typically tumor cell lines such as RPMI8866, Epstein-Barr virus lymphoblastoid cell line, and K562, significantly enhances NK cell expansion. Coculture systems involving irradiated feeder cells and NK cells in media supplemented with IL-2 or IL-15 have been developed to generate large quantities of NK cells [14]. Feeder cells are genetically modified to express specific proteins capable of expanding, activating, and increasing the cytotoxicity of NK

cells through the insertion of exogenous proteins via lentivirus.

Notably, Dr Campana's group discovered that K562 cells coexpressing membrane-bound IL-15 and 4-1BBL act synergistically to enhance their stimulatory capacity for NK cells [15]. This results in robust expansion of $CD56^{+}CD3^{-}$ NK cells from peripheral blood without a concomitant increase in T lymphocytes [15-18].

The development of feeder cells for ex vivo expansion of NK cells is a crucial aspect of NK cell therapy. Feeder cells play a significant role in enhancing the expansion of NK cells and enabling the effective application of NK cell therapy. This process provide essential growth factors and support for the expansion and maintenance of NK cells in the laboratory [14].

The cocultivation of NK cells with feeder cells plays a pivotal role in the development of this type of therapy. However, despite the well-established nature of this concept, centers that develop their own feeder cells often refrain from sharing their products because of intellectual property concerns, such as patents.

Taking inspiration from the successful work of Lee et al. [19] and Ojo et al. [20], esteemed groups that have achieved the production of their own feeder cells, our study aimed to develop a feeder cell that would be readily accessible within our territory. To accomplish this, we transduced the K562 cell line with mbIL-21 and 4-1BBL, with the specific objective of constructing a feeder cell that could effectively promote the proliferation of NK cells while simultaneously preserving their cytotoxicity and preventing senescence or exhaustion. Through our research, we aimed to make a meaningful contribution to the advancement of NK cell therapy and provide a valuable resource at a national level.

METHODS

Isolation of Human NK Cells and Ethical Aspects

All study procedures were performed in accordance with the Declaration of Helsinki. The Research Ethics Committee of the Hospital Israelita Albert Einstein approved the study (protocol 06191819.9.0000.0071).

Peripheral blood was collected from 5 healthy donors to obtain NK cells and from 1 patient with acute myeloid leukemia to acquire blasts for the cytotoxicity test. All donors provided informed written consent to participate in the study. The collection occurred at the Hospital Israelita Albert Einstein from August 11, 2021, to March 9, 2022.

Production of Lentiviral Vectors Containing mIL-21 or 4-1BBL

The lentiviral vectors were produced using an HEK293 T expression system. In brief, HEK293T cells were transfected with packing plasmid psPAX2 (clone 12260; Addgene), envelope plasmid pmD2.G (12259; Addgene) and plasmids carrying on the sequence of mbIL-21 (Vector Builder, pLV and [Exp]-Puro-EF1A>membrane bound IL-21 [mbIL-21]) or expressing the sequence of 4-1BBL (Vector Builder, pLV[Exp]-Hygro-EF1A>4-1BBL) using X-tremeGENEHP DNA Transfection Reagent (Merck).

K562.Clone1 Feeder Cell Manufacture

K562 cells (CCL-243-ATCC) were obtained from Rio de Janeiro Cell Bank by Dr Ricardo Weinlich (Hospital Albert Einstein). The cell identity was confirmed through K562 STR DNA fingerprinting (https://www.atcc.org/products/ccl-243) using a GenePrint 10 System Kit (Promega).

K562 feeder cells were transduced with lentiviral vectors containing genes encoding mbIL-21 or 4-1BBL, along with 8 μg/mL of Polybrene (hexadimethrine bromide; Sigma-Aldrich), following the protocol established by Dr Wald at Case Western Reserve University [20]. As a result of this transduction, a K562.Clone1 cell line that expresses mbIL-21 and 4-1BBL on its surface was generated.

All clones expressing mbIL-21 and/or 4-1BBL were initially selected using antibiotics. However, to further enhance purity, the K562.Clone1 cell line was sorted using the MoFlo Astrios EQ cell sorter (Beckman Coulter).

The feeder cells were expanded using RPMI 1640 medium supplemented with 10% inactive bovine fetal serum, 1% L-glutamine, and 1% penicillin and streptomycin (Gibco). Before coculturing with peripheral blood mononuclear cells (PBMCs) or NK cells, the K562.Clone1 cells were irradiated at a dose of 1 Gy using the GamaCell3000 irradiation system (Best Theratronics) [21].

Confocal Analysis to Evaluate the Expression of mbIL-21 and 4-1BBL on K562.Clone1 Cells

K562.Clone1 cells were plated in a round-bottom 96-well plate at a density of 3.0×10^{5} cells per well. The cells were washed and resuspended in 100 μL of phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (buffer). Specific antibodies against mbIL-21 (clone BV421; BD Biosciences; 3.5 μL) and 4-1BBL (4-1BBL PE; 1.75 μL) were used for cell labeling. The labeling

process was conducted for 30 minutes at room temperature.

Following the labeling step, the cells were washed and fixed in a 1% formaldehyde solution diluted in buffer. The fixed cells were then transferred to a flat-bottom 96-well plate and stored at 4°C until further analysis.

The same procedure was performed with K562-WT cells for comparison purposes. This experimental approach facilitated the visualization and analysis of mbIL-21 and 4-1BBL expression patterns on K562.Clone1 cells using confocal microscopy. This provided valuable insight into the localization and distribution of these molecules on the cell surface. Confocal microscopy imaging was performed with a Zeiss LSM710 confocal microscope aided by Dr Thiago Aloia, a research assistant. K562.Clone1 was labeled with the same antibodies for 30 minutes, and acquired in flow cytometer to complement the confocal images.

NK Cell Expansion

PBMCs were isolated using density gradient centrifugation (Ficoll Paque Plus; GE Health). The isolated PBMCs from a single donor were then cocultured in G-rex flasks (Wilson Wolf) with irradiated K562.Clone1 cells or irradiated K562-WT cells, which served as a control. The use of PBMCs from the same donor in both coculture conditions aimed to minimize inter-donor variability, allowing for a more controlled comparison between the experimental and control groups. This approach enhances the accuracy of assessing specific effects resulting from the interaction of PBMCs with either K562.Clone1 cells or control K562-WT cells.

On day 7 of the coculture, the CD3 fraction of the cells was depleted using an LD column (130-050-101; Miltenyi Biotec). The CD3-negative portions were then cocultured with irradiated K562. Clone1 cells or irradiated K562-WT cells in G-rex flasks at a maintained ratio of 1:4 throughout the experiment.

To support growth and activation of the cocultured cells, 100 U/mL of IL-2 (Gibco) was added to the culture every 2 days. IL-2 is known for its role in promoting cell expansion and function. Additionally, irradiated feeder cells or irradiated K562-WT cells were added to the coculture on days 0, 7, and 14. This continuous supply of stimuli and support ensured a favorable environment for the cocultured cells throughout the experimental period, facilitating their viability and optimal growth.

Characterization of ex Vivo Expanded NK Cells

Immunophenotyping analysis was conducted on days 0, 7, and 14 to characterize the cell populations. The cells were incubated with monoclonal antibodies and labeled with specific markers to assess their phenotype and functional properties for 30 minutes and then washed twice with FACS buffer (PBS + .1% BSA) to remove unbound antibodies.

NK cells were characterized using the following antibodies: CD56 (clone NCAM16.2; BD Biosciences), CD16 (clone 3G8; BD Biosciences), Live/Dead Fixable Aqua Dead Cell Viability Reagent (405-nm excitation; clone L34957; Invitrogen), anti-TCR-$\gamma\delta$ (clone B1; BD Biosciences), anti-CD3 (clone SK7; BD Biosciences), CD45 (clone HI30; BD Biosciences) [22-24].

NK activator and inhibitory receptors were evaluated using the following antibodies: NKG2C (clone 134591; BD Biosciences), NKG2D (clone 1D11; BD Biosciences), DNAM-1 (clone 11A8; BD Biosciences), NKp30 (clone 210845; R&D Systems), NKp44 (clone P44-8; BD Biosciences), NKG2A (clone 131411; BD Biosciences), KIR3DL1 (clone DX9; Biolegend), KIR2DL2/3 (clone DX27, Biolegend), and KIR2DL1 (clone HP-MA4; Biolegend) [22,24,25].

For cytotoxic marker evaluation, intracellular staining was performed. NK cells were first labeled with CD107a (clone H4A3; Biolegend) and incubated with FIX & PERM Cell Permeabilization reagent (Invitrogen), according to the manufacturer's instructions. After the permeabilization step, the NK cells were incubated with specific antibodies, including Perforin (clone δG9; BD Biosciences), TNF-α (clone MAB11; Biolegend), IFN-γ (clone B27; BD Biosciences), Granzyme A (clone CB9; Biolegend), and Granzyme B (GB11; BD Biosciences) for 20 minutes. Following incubation, the cells were washed to remove any remaining unbound antibodies [18,26-28].

For senescence and exhaustion evaluation, the following antibodies were used: anti-PD-1 (clone EH12.2H7; Biolegend), anti-CD57 (clone NK-1; BD Biosciences), anti-TRAIL (clone RIK-2; BD Biosciences), anti-TIM-3 (clone 7D3; BD Biosciences), anti-KI-67 (clone B56; BD Biosciences), anti-EOMES (clone WD1928; Ebioscience/Thermo Fisher Scientific), and anti-T-BET (clone 4B10; Biolegend) [25,29-31].

Flow cytometry analysis was performed using either the BD LSRFortessa (BD Biosciences) or Attune NxT (Thermo Fisher Scientific) flow cytometers. Data acquisition was performed, and the acquired data were analyzed using Flowjo 10 software (BD Biosciences).

Cytotoxicity Assay

NK cell cytotoxic function was assessed using the calcein-AM (CAM) assay. The target tumor cells used in the assay included K562 (human erythroleukemia lineage), OCI-AML2 (acute myeloid leukemia [AML] lineage), AML Blast (from a patient diagnosed with AML), and U-87 MG (glioblastoma lineage, kindly provided by Dr Keith Okamoto, University of São Paulo).

In the CAM assay, target cells were labeled with 25 nM of calcein-AM for 30 minutes and then washed twice with PBS supplemented with 5% inactive fetal bovine serum. The labeled target cells were resuspended in complete medium at a concentration of 1×10^4 cells/mL.

Labeled target cells were plated in triplicate in 96-well round bottom microwell plates (Corning), with NK:T cell ratios ranging from .5:1 to 40:1. The controls included nonstained target cells, CAM-stained cells, and labeled target cells in medium plus 2% Triton X-100. The assay plates were then incubated at 37 °C in a 5% CO_2 environment for 4 hours.

Following the incubation period, the samples were acquired with either an LSR Fortessa (BD Biosciences) or an Attune (Thermo Fisher Scientific) flow cytometer. The percent cell lysis was calculated as described by Ojo et al. [20].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software. The data are presented as mean ± standard deviation (range). The differences between groups were determined using 2-way or 3-way analysis of variance (ANOVA), as appropriate. Multiple *t* tests were used to evaluate ratios or markers. The Wilcoxon signed-rank test was used to compare the medians of samples, considering a hypothetical value of 0 used in the calcein assay. A P value = .05 was considered statistically significant, and denotations also were provided for comparisons where $P < .01$ and $P < .001$. Graphics were plotted with Microsoft Excel.

RESULTS

Expression of mbIL-21 and 4-1BBL on K562.Clone1 Cells

Successful transduction of cells was confirmed through the confocal microscopy assay. The expression levels of both 4-1BBL and mbIL-21 were significantly higher in transduced cells—K562.Clone1—compared to K562-WT cells (Figure 1A). The analysis of median fluorescence intensity (MFI), performed using Zen lite version 3.4, revealed an MFI of 28,000 for 4-1BBL in K562.Clone1 cells, whereas K562-WT cells had an MFI of 4,000. Additionally, K562.Clone1 cells exhibited an MFI of 372,000 for mbIL-21, whereas K562-WT cells had an MFI of 18,000.

To complement the data obtained from confocal microscopy, we acquired the feeder cell K562.Clone1 on a flow cytometer, using the same selected cells. The analysis was performed with FlowJo. The strategic analyses were gated cell populations based on morphology, excluding doublets, selecting viable cells, and quantifying 4-1BBL and mbIL-21. Notably, both markers showed nearly 100% expression of these proteins (Figure 1B).

The first test compared the expansion rate of K562.Clone 1 and IL-2 using fresh PBMCs. The second test compared K562.Clone1 and K562-WT using the same donor sample, but this time starting from a frozen sample. The results indicated that regardless of whether the sample was cocultured with K562.Clone1, the cells had the ability to expand significantly. In the fresh sample, the cell count increased from 2.0×10^6 cells to 172.40×10^6 NK cells, an approximate 87-fold increase, whereas in the frozen sample, the cell count increased from 2 to 131.40×10^6 NK cells, indicating an approximate 66-fold expansion (Figure 2A).

When IL-2 was used without any feeder cell, the cell count reached 19.66×10^6 NK cells, even though the sample was fresh. Similarly, when K562-WT was used, the cell count was 21.45×10^6 NK cells over the same 14-day period (Figure 2A). This indicates a nearly 10-fold expansion in both cases.

Owing to the superior expansion observed using fresh samples, we proceeded to conduct further assays using the samples in their fresh state. When comparing the expansion rate of NK cells from day 0 to day 14 using fresh PBMCs from each of the 5 donors, a significant difference was observed depending on whether K562.Clone1 or K562-WT was used. In the presence of K562.Clone1, the median expansion was 186.60×10^6 NK cells (range, 81.30 to 308.25×10^6), representing an approximate 94-fold expansion of NK cells. Conversely, with K562-WT, the median expansion was 34.05×10^6 NK cells (range, 10.60 to 91.00×10^6), resulting in a median 17-fold expansion (Figure 2B). These findings provide compelling evidence for the superior ability of K562.Clone1 to enhance the expansion of NK cells compared to K562-WT.

It is noteworthy that the initial culture contained a median of 9.56% (range, 6.26% to 12.3%)

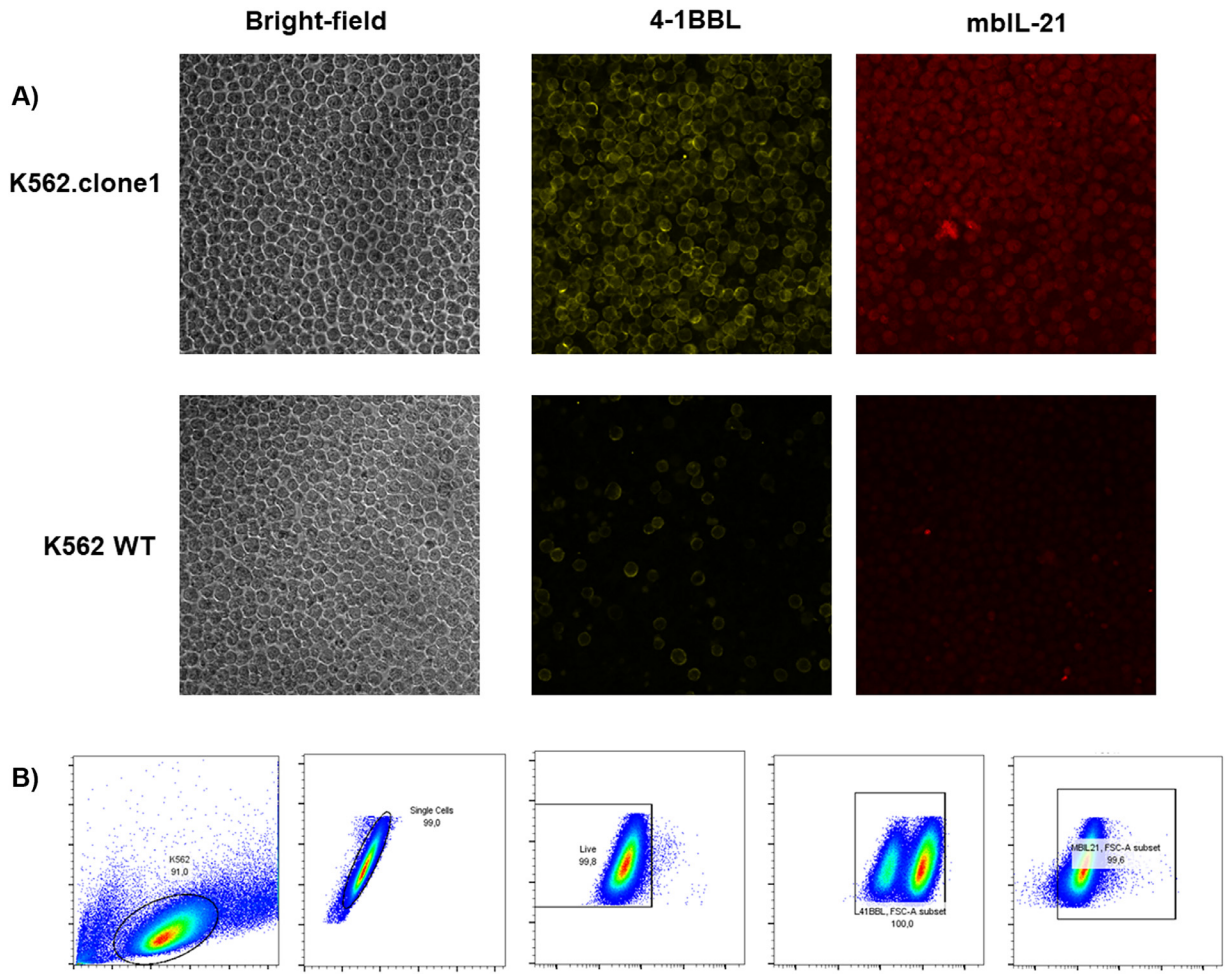

Figure 1. Confocal microscopy and flow cytometry analysis of 4-1BBL and mbIL21 expression in K562.Clone1 and K562-WT cells. (A) Confocal microscopy images clearly show higher MFI in K562.Clone1 cells compared to K562-WT cells, while maintaining the preserved morphology of K562 cells. (B) Flow cytometry analysis confirms the expression of 4-1BBL and mbIL-21 in both cell types.

of NK cells ($CD3^-CD56^+$). However, by day 14, there was a significant increase in the population of NK cells, accounting for approximately 96% of the total cell count (range, 92.90% to 99.40%) (Figure 2C). This substantial enrichment of the NK cell population in the culture, achieved through the use of K562.Clone1, highlights the efficacy of this approach.

NK Cell Cytotoxic Evaluation

We conducted an analysis on D14 NK cells to examine the expression of CD107a, granzyme A, granzyme B, INF-γ, perforin, and TNF-α. Unlike the previous analyses, which focused primarily on evaluating the quantity of NK cells, this cytotoxicity analysis aimed to investigate the NK^{dim} ($CD45^+CD3^-CD56^+CD16^+$) and NK^{bright} ($CD45^+CD3^-CD56^+CD16^-$) subpopulations. This differentiation was deemed important, as NK^{bright} cells are primarily responsible for producing cytokines, which play a crucial role in regulating immune responses, whereas NK^{dim} cells have cytotoxic capabilities, enabling direct killing of target cells. By considering both subsets, the potential effectiveness of immunotherapy products in combating tumors can be enhanced significantly [32].

The median and SD of each cytotoxic marker is depicted in Figure 3A. Three-way ANOVA performed to evaluate the difference between NK cell coculture with K562.Clone1 (F-NK) or with K562-WT (WT-NK) on the expression of cytotoxic markers revealed a statistically significant difference between the cytotoxic markers expressed by F-NK and WT-NK cells ($P < .05$).

Using *t*-distributed stochastic neighbor embedding (t-SNE), we visualized the cytotoxic expression profiles of CD56, CD16, CD107a, granzyme A and B, IFN-γ, perforin, and TNF-α (Figure 3B). The t-SNE analysis effectively clustered cells based on

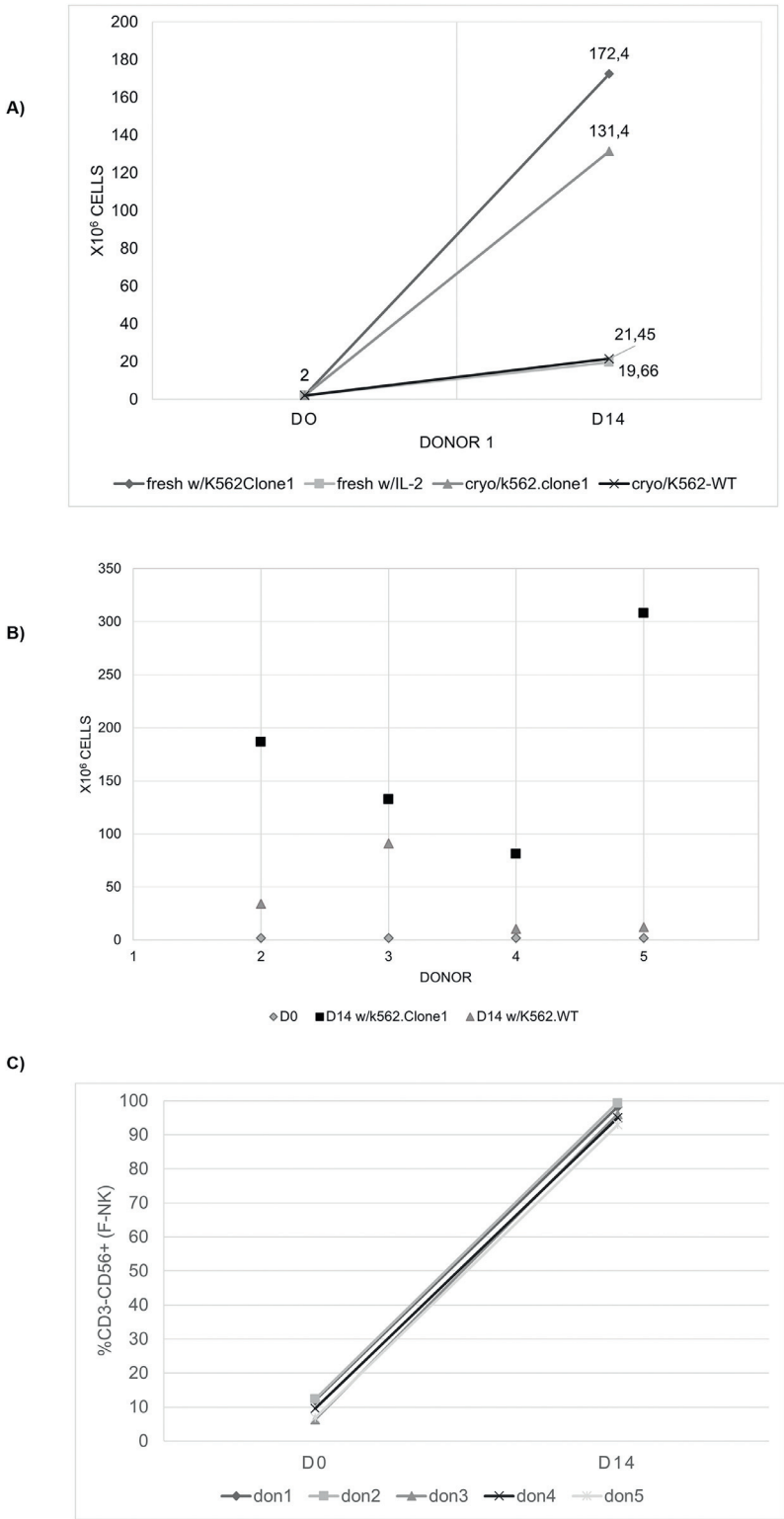

Figure 2. Enhanced NK cell expansion and expression with K562.Clone1 compared to IL-2 or K562-WT. (A) NK cell expansion rates of K562.Clone1 were compared to IL-2 and K562-WT. Coculturing with K562.Clone1 resulted in significantly higher cell expansion levels compared to IL-2 or K562-WT, whether fresh or cryopreserved. (B) NK cell expansion rates were significantly higher with K562.Clone1 (median, 186.60 $\times$ 10^6; range, 81.30 to 308.25 $\times$ 10^6) compared to K562-WT (median, 34.05 $\times$ 10^6; range, 10.60 to 91.00 $\times$ 10^6). The initial culture had a median NK cell percentage of 9.56% (range, 6.26% to 12.3%). **(**C) NK cell expression ($CD3^-CD56^+$) was evaluated at day 0 and day 14. The NK cell percentage at the start of the culture was 9.56% (range, 6.26% to 12.3%), increasing to 96% (range, 92.90% to 99.40%) by day 14, indicating a significant expansion of NK cells.

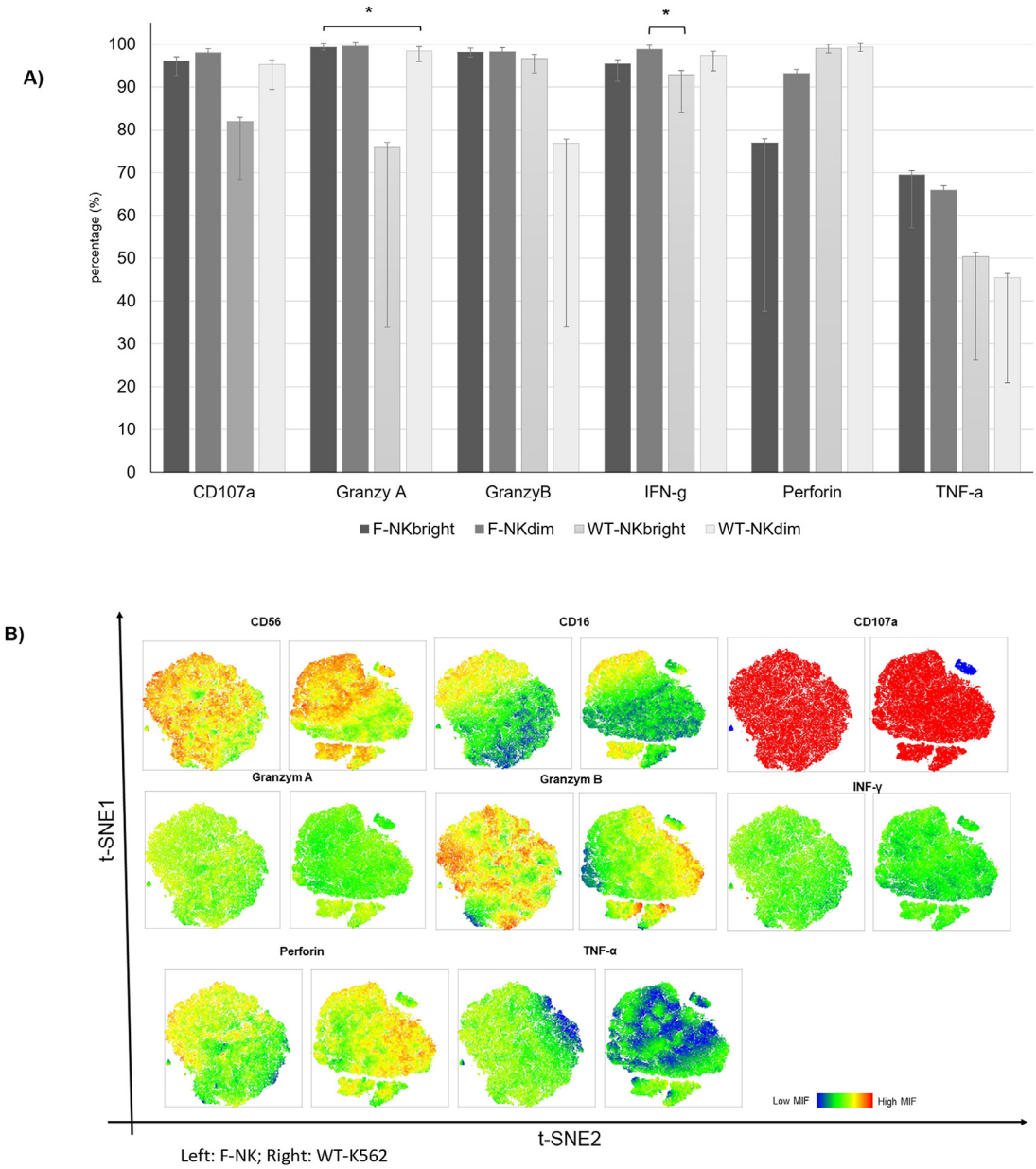

Figure 3. Cytotoxic marker evaluation in F-NK and WT-NK cells. (A) The bar chart shows the mean and SD of CD107a, granzyme A (GranzyA), granzyme B (GranzyB), IFN-γ (IFN-g), perforin, and TNF-α (TNF-a). NK cells cocultured with K562.Clone1 (dark gray) exhibit significantly higher expression levels compared to those cocultured with K562-WT (light gray), as indicated by a 3-way ANOVA. *$P < .05$. (B) t-SNE analysis shows cytotoxic expression profiles of CD56, CD16, CD107a, GranzyA/B, IFN-g, perforin, and TNF-a. The distribution of NK cell populations is influenced by feeder cells, with 2 clusters observed for K562.Clone1 cocultures and 4 clusters for K562-WT cocultures. Notably, F-NK cells exhibit higher MFI and thus greater cytotoxic potency compared to WT-NK cells.

their similarities, revealing that cocultured cells with K562.Clone1 feeder cells formed 2 clusters, whereas those with K562-WT feeder cells formed 4 clusters. These findings suggest that cells cocultured with F-NK exhibit a more homogeneous maturation and expansion compared to cells cocultured with WT-NK.

The presence of CD107a in both populations indicates the ability of NK cells to degranulate in the presence of a tumor target. The analysis also reveals the presence of granules containing granzyme A and B, IFN-γ, perforin, and TNF-α, which are important for mediating cytotoxicity.

Granzyme A and B are proteolytic enzymes involved in cell-mediated immune responses, with the ability to induce programmed cell death in target cells. Each of them has specific mechanisms of action, contributing to the effective elimination of infected or tumor cells. The main function of granzyme A is to induce apoptosis by

activating caspases, which are enzymes responsible for the degradation of cellular proteins and the induction of programmed cell death. In addition, granzyme A can also modulate the immune response and have anti-inflammatory effects [33].

On the other hand, granzyme B is known for its ability to induce cell death through different mechanisms. It can directly activate caspases, but it also can trigger the release of cytochrome C from mitochondria, initiating a cascade of events that lead to apoptosis. Additionally, granzyme B can cause direct damage to target cells, such as the cleavage of structural proteins [33,34].

When NK cells encounter tumor cells, they can secrete interferons, such as IFN-γ. IFN-γ has various functions in the antitumor immune response; it can enhance the cytotoxic activity of NK cells and cytotoxic T cells, promoting the death of tumor cells. Furthermore, IFN-γ can modulate the immune response by stimulating antigen presentation by antigen-presenting cells and increasing the expression of major histocompatibility complex class I molecules on tumor cells, facilitating the recognition and elimination of tumor cells by T cells [35].

Perforin is a protein that plays a fundamental role in the function of NK cells. The main function of perforin is to induce the death of target cells by NK cells. When NK cells identify a target cell, they release cytotoxic granules containing perforin. Perforin then forms pores in the target cell's membrane, allowing the entry of cytotoxic enzymes, such as granzymes, into the target cell. These enzymes trigger a cascade of events that result in the death of the target cell [36].

An important cytokine is TNF-α, which induces apoptosis (programmed cell death) in tumor cells, effectively inhibiting their growth and spread. TNF-α also exhibits immunomodulatory properties, meaning that it can modulate the activity of other immune cells. It enhances the expression of adhesion molecules on endothelial cells, facilitating the migration of other immune cells to the site of infection or inflammation. This allows for a coordinated immune response against the threat [37].

Moreover, TNF-α stimulates the production of other inflammatory cytokines, such as IFN-γ, further contributing to the immune response. IFN-γ plays a vital role in regulating immune cell activity and promoting the elimination of infected or damaged cells [38]. In summary, the analysis of high-dimensional cytotoxic expression profiles and the visualization of data using t-SNE allow us to better understand the behavior of NK cells and their cytotoxic capacity [39]. These cells can recognize and eliminating tumor cells using a variety of mechanisms, such as degranulation, release of cytotoxic enzymes, and production of proinflammatory cytokines.

The analysis of high-dimensional cytotoxic expression profiles and the visualization of data using t-SNE allow us to gain a better understanding of the behavior and cytotoxic capacity of NK cells. These cells use various mechanisms, including degranulation, release of cytotoxic enzymes, and production of proinflammatory cytokines, to recognize and eliminate tumor cells. The interconnected actions of granzyme A and B, perforin, interferons, and TNF-α contribute to the effective immune response against tumor cells, ultimately leading to their elimination.

The cytotoxic potency assay confirmed the cytotoxic efficiency of F-NK and WT-NK cells against various cell lineages, including chronic myeloid leukemia (K562), blasts from AML patients, OCI-AML2 (AML lineage), and glioblastoma (U-86MG). The calcein-AM assay was performed on day 14 of the experiment, with triton-treated samples serving as positive controls [20,40]. This assay measures the potency of F-NK and WT-NK cells across the range of effector (NK) versus target cell ratios, ranging from .5:1 to 40:1. The results revealed that both F-NK and WT-NK cells were effective across a range of effector-to-target ratios. Specifically, F-NK cells demonstrated high killing ratios, achieving 65.07% (range, 36.20% to 85.24%) against OCI-AML2, 89.19% (range, 82.95% to 92.99%) against K562, and 51.23% (range, 43.03% to 62.84%) against AML blasts from a patient with AML (Figure 4A-C).

Similarly, WT-NK cells displayed a median cytotoxicity of 57.40% against OCI-AML2 (range, 22.29% to 82.82%), 87.74% against K562 (range, 75.72% to 92.95%), and 54.66% against AML blasts (range, 30.28% to 70.73%). The Wilcoxon rank-sum test was used to compare the cytotoxic potency of F-NK and WT-NK cells (Figure 4A-C). The results indicated a statistically significant difference between the culture conditions ($P < .05$). Statistical analysis of the U87MG (glioblastoma cell line) provided valuable insight into the observed differences at 2 specific ratios, 10:1 and 40:1 ($P < .05$). F-NK cells demonstrated a high killing ratio, achieving an average cytotoxicity of 67.45% (range, 58.60% to 73.81%), whereas WT-NK cells displayed a median cytotoxicity of 33.32% against U87MG (range, 21.15% to 40.90%). These findings suggest that F-NK cells have a greater capacity to eliminate

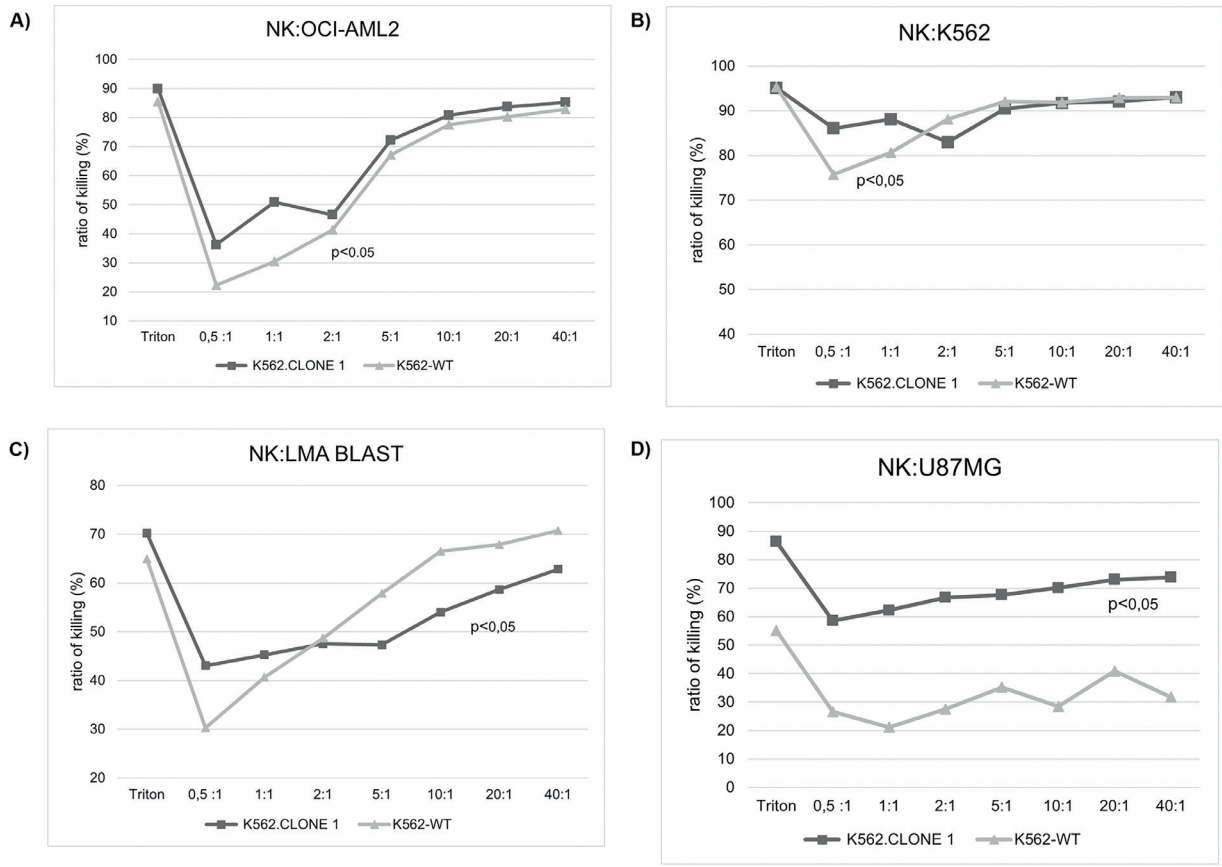

Figure 4. Functional assays in NK cells. Different ratios of NK cells ranging from .5:1 to 40:1 were used to measure their cytotoxic potency against various leukemia cell lines, including K562 (A), OCI-AML2 (B), LMA Blast (C), and glioblastoma (D). The results of the analysis using 3-way ANOVA indicate a statistically significant difference in cytotoxic markers between NK cells expanded with K562.Clone1 (triangle) and NK cells expanded with K562-WT (square). *$P < .05$.

U87MG cells compared to WT-NK cells (Figure 4D).

NK Cell Fitting

The balance between activatory and inhibitory receptors refers to the delicate equilibrium between the receptors present on the surface of NK cells that promote their activation and those that inhibit their activity. Activatory receptors recognize specific molecules on target cells, triggering the cytotoxic function of NK cells, while inhibitory receptors interact with molecules on healthy cells, preventing NK cells from attacking them [41]. Maintaining this balance is essential for the optimal functioning of NK cells in immune responses.

In this way, D14-NK cell coculture with K562. Clone1 or WT-K562 were labeled with activator and inhibitory markers and then acquired in flow cytometer. The NK^{dim} and NK^{bright} populations exhibited a balanced distribution of activation and inhibition markers (Figure 5A, B). This proteins NKG2D, NKp30, and NKp44 were very similar in F-NK and WT-NK cells, regardless of the subpopulation (Figure 5C). DNAM-1 expression was not detected in any of our samples. Notably, a significant difference was observed in the expression of the NKG2C activation receptor between the NK^{dim} and NK^{bright} subpopulations, particularly in the context of coculture with K562.Clone1 ($P < .05$, 3-way ANOVA), this data could be related to more mature and specialized immune responses after exposed an antigen-experienced [42].

Regarding inhibition receptors such as KIRs and NKG2A, Figure 5D demonstrates distinct means between NK^{dim} and NK^{bright} populations. The statistical analysis revealed a significant difference in the distribution of these receptors between the growth-supporting cells used in the coculture ($P < .005$). The mb-IL21 and 4-1BBL affect positively cell growth by providing a microenvironment that modulates the expression of these receptors [21,43].

As observed, NK cells cultured with K562. Clone1 maintained the expression of CD56 and CD16, the most important phenotypic markers. Moreover, these cells demonstrated potent

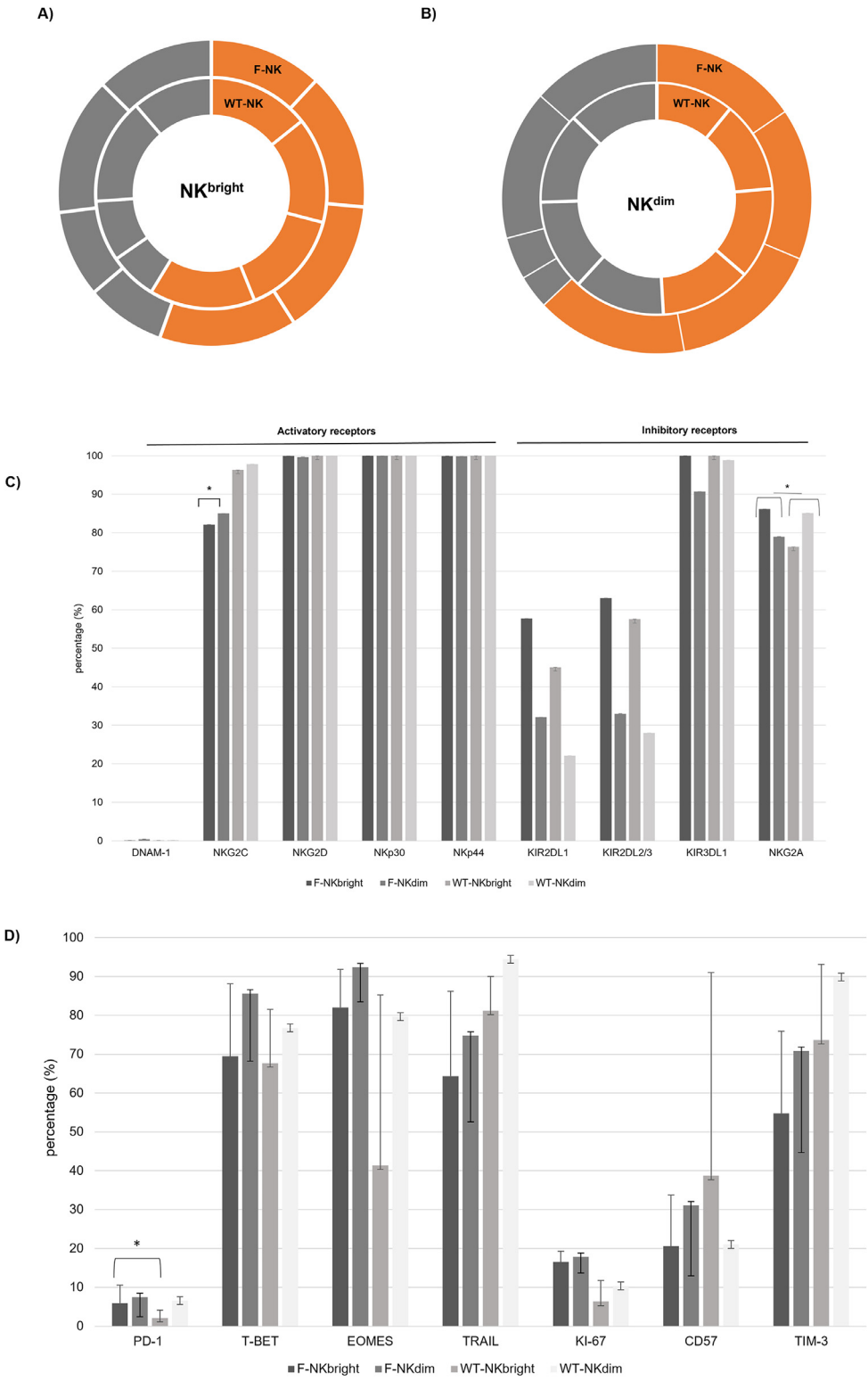

Figure 5. Activator and inhibitor markers and NK cell balance. (A and B) Expression of activator NK cell markers (DNAM-1, NKG2C, NKG2D, NKp30, and NKp44) and inhibitor markers (KIR2DL1, KIR2DL2/3, KIR2DL1, and NKG2A) in different NK cell subpopulations cocultured with either K562.Clone1 (F-NK) or K562-WT cells (WT-NK), highlighting the differential expression patterns associated with these coculture conditions. (C) NK cells expanded with K562.Clone1 feeder cells show low expression of the exhaustion marker PD-1, indicating the absence of exhaustion. Additionally, these expanded NK cells exhibit active cell division, as indicated by the presence of the proliferation marker KI-67. Furthermore, markers associated with cell maturation, such as CD57 and TIM-3, are observed in these NK cells. The high expression levels of T-BET, EOMES, TRAIL, and other cytotoxic factors further suggest a functional and mature state of these NK cells. These findings collectively demonstrate the favorable phenotype and functional potential of NK cells cocultured with K562.Clone1 compared to K562-WT cells.

cytotoxic activity (Figure 4C), probably by releasing granules containing interferons or granzymes (Figure 3), important for promoting apoptosis on tumor cells [44]. It also is noteworthy that NK cells cocultured with K562.Clone1 were not senescent or exhausted, indicating that these cells maintained their functional integrity and remained responsive to their targets, highlighting their potential efficacy in immune responses.

To comprehensively evaluate the state of anergy, senescence, and cellular exhaustion, we conducted a thorough assessment of various markers including KI-67, which indicates cell proliferation; CD57 and TIM-3, which serve as indicators of maturational stage; TRAIL (CD253), a ligand associated with apoptosis induction; PD-1, a marker of exhaustion; and transcription factors, such as T-bet and eomesodermin (EOMES), which are linked to activation and inhibition markers (Figure 5D) [29,45-48].

Our findings indicate that NK cells exhibited preserved cytotoxic ability, as evidenced by the low expression of PD-1 and high expression of T-BET, EOMES, and TRAIL (Figure 5D). These results suggest that the NK cells were not senescent or exhausted. Moreover, the expression of KI-67 suggested that F-NK cells have a higher proliferation rate than WT-NK cells and are associated with CD57 and TIM-3 expression, indicating that the F-NK cells had reached a mature stage. Statistical analyses revealed significant differences in the expression of these markers between the $CD56^{bright}$ and $CD56^{dim}$ populations, regardless of the coculture used ($P < .05$; 3-way ANOVA).

DISCUSSION

NK cells have innate cytotoxicity toward tumor cells and compose approximately 10% to 15% of peripheral blood lymphocytes in healthy individuals. In cord blood, they constitute roughly 25% to 30% of the lymphocytes present [49,50]. Unlike T cells and B cells, NK cells have a unique ability to identify and eliminate various abnormal cells without the need for prior sensitization or recognition of specific antigens [51,52].

NK cells accomplish this by detecting the absence of "self" markers, specifically MHC-I molecules, on cancer cells and virus-infected cells. This recognition, commonly known as the "missing self" hypothesis, empowers NK cells to identify and eliminate these cells, as well as target cells that display heightened expression of stress-induced ligands or an up-regulation of death receptor [53,54].

Additionally, NK cells play a crucial role in hematopoietic stem cell transplantation (HSCT) by improving clinical outcomes through lower relapse rates and reduced incidence of graft versus host disease (GVHD) [2,55]. NK cells also play a role in preventing GVHD, which is a potentially life-threatening complication that can occur after transplantation. They possess the ability to regulate the activity of other immune cells, such as T cells, and help maintain a delicate balance between effectively attacking cancer cells while avoiding an excessive immune response against healthy tissues [56].

Moreover, the infusion of NK cells derived from a haploidentical donor who is partially matched with the recipient has shown promise as a therapeutic approach in HSCT. This approach aims to enhance clinical outcomes in the context of transplantation. Additionally, adoptive transfer of NK cells, which involves collecting, activating, or expanding NK cells before reintroducing them into the patient, has emerged as a promising therapeutic strategy for various types of cancer, including AML and multiple myeloma. These advances highlight the potential of NK cells in improving treatment outcomes for these diseases [57,58].

A phase I/II clinical trial conducted by Ciurea et al. [59] provided evidence of a positive association between adoptive immunotherapy using ex vivo expanded NK cells with feeder cells and the clinical outcomes of AML patients undergoing HSCT. The trial included a follow-up of 24 months, during which the relapse rate was found to be significantly lower in the group receiving NK adoptive immunotherapy compared to the group receiving conventional treatment (4% versus 38%). Additionally, the disease-free survival rate was higher in the NK adoptive immunotherapy group compared to the control group (66% versus 44%). The trial also revealed that approximately 50% of the AML patients, that receive NK dose, experienced grade I-II acute graft-versus-host disease (aGVHD), with rare cases of grade III-IV aGVHD. No cases of chronic GVHD were observed during the follow-up period in patients treated with NK adoptive immunotherapy. These findings indicate the potential of NK cell therapy to mitigate the occurrence of GVHD in AML patients undergoing HSCT [12,59].

The study also investigated the impact of different NK cell doses administered post-transplantation. It was found that higher doses of NK cells (1×10^8 cells/kg) can lead to early immune

reconstitution, reducing the risk of relapse and extending the disease-free period [12,59].

In a separate clinical study conducted by Otegbeye et al. [60], the efficacy and safety of ex vivo expanded NK cells were evaluated in patients with myelodysplastic syndrome, AML, or colorectal cancer. Prior to receiving NK adoptive immunotherapy, these patients had undergone chemotherapy with cyclophosphamide and fludarabine. The study involved the administration of 2 NK cell doses ranging from 1×10^7 cells/kg to 5×10^7 cells/kg. The study also emphasized the minimal toxicity associated with the doses of NK cells administered, reaffirming the safety profile of this therapy in cancer patients.

The field of NK cell research is advancing rapidly, with several ongoing clinical studies exploring chimeric antigen receptors in NK cells, the modifications aim to promote targeted responses against cancer cells. The engineering of CAR NK cells, involves a strategy similar to that for CAR T cells, involving the use of extracellular receptor scFV for HLA-unrestrictive antigen recognition. Another strategy is to produce a functional CD3/TCR signaling complex to recognize MHC-I, which potentially could allow for the use of NK cells to produce off-the-shelf products. Another way to redirect NK cell specificity is to use bispecific or trispecific antibodies to induce tumor cell death via CD16 through a process of antibody-mediated cytotoxicity; such multiantigen targeting shows a favorable safety profile [8,61]. Another promising approach involves the use of interleukin cocktails to induce a memory in NK cells, resulting in the generation of potent NK memory-like cells that can respond effectively against lymphoma [61,62].

The findings from the aforementioned studies provide strong support for the efficacy, safety, and potential of adoptive immunotherapy using ex vivo expanded NK cells in the management of various cancers, including AML, myelodysplastic syndrome, and colorectal cancer [53,63,64]. These studies highlight the significant advancements in NK cell therapy and its potential to improve treatment outcomes.

Feeder cells play a critical role in the ex vivo expansion process of NK cells, as evidenced by the findings of Denman et al. [21] and Vidard et al. [51]. Vidard et al. found that nontransduced K562 cells exhibited robust initial expansion but lacked the ability to sustain prolonged proliferative responses owing to the absence of certain factors, such as 4-1BBL and mbIL-21. In contrast, Denman et al. demonstrated the effectiveness of their ex vivo expansion platform using feeder cells, which successfully supported the clonal expansion of NK cells. This platform not only maintained the viability of the cells, but also enhanced their antitumor cytotoxicity.

These collective findings emphasize the significance of feeder cells in optimizing the ex vivo expansion of NK cells. The use of specific stimulation methods, optimized culture conditions, and appropriate selection of feeder cells significantly impact the yield, functionality, and cytotoxic efficacy of expanded NK cells. This optimization is crucial for the development of innovative treatment options that cater to the specific needs of patients. For instance, Fujisaki et al. [65] generated a K562-mb15-41BBL feeder cell that expanded NK cells by an average of 21.6-fold within the first 7 days of coculture, and Jiang et al. [43] produced a K562.4-1BBL feeder cell capable of expanding NK cells by up to 1000-fold after 21 days. Denman et al. [21] used feeder cells based on mbIL21, 4-1BB, and CD86, achieving NK cell expansion ranging from 31.74 to 47.96 folds after 21 days of co-culture.

In our study, the K562.Clone1 feeder cell exhibited the remarkable capacity to expand NK cells by up to 100-fold, depending on the donor source, while simultaneously preserving their phenotypic features and cytotoxic function. Notably, these expanded NK cells showed no signs of exhaustion or senescence, demonstrating their potential for therapeutic applications, and the balance between NK^{dim} and NK^{bright} subsets has a crucial role in maintaining a well-functioning immune response.

Particularly in the context of immunotherapy, the presence of both subsets can prove highly advantageous. NK^{bright} cells are renowned for their ability to produce cytokines, which serve to stimulate the immune system and facilitate coordinated responses against cancer cells. In contrast, NK^{dim} cells have remarkable cytotoxic capabilities, enabling them to directly eliminate target cells, including cancer cells. By combining both subsets, immunotherapy products can effectively harness the benefits of cytokine production and direct cytotoxicity, thereby enhancing their overall effectiveness [32,66].

Nevertheless, NK cells expanded with feeder cell K562.Clone1 exhibited active cytotoxicity, as evidenced by the expression of CD107a, an important protein involved in the degranulation ability of NK cells. This protein is a glycosylated molecule that preserves the integrity of the lysosomal membrane and degranulates only when encountering a tumor cell [67]. These granules contain

cytolytic factors, such as perforin and granzyme, responsible for direct lysing of the tumor cell with induction of IFN-γ and TNF-α, for example, when an NK cell recognizes a cancer cell [29,68].

When an NK cell recognizes a target cell, it forms a synapse with the target cell, known as the immunologic synapse. This synapse allows for the transfer of signals and the release of cytotoxic granules. For example, perforin is a protein that creates channels that allow the entry of granzymes into the target cell, inducing apoptosis, or programmed cell death. Once inside the target cell, granzymes can activate caspases, enzymes responsible for initiating the apoptotic process [69].

In addition to perforin and granzymes, NK cells may release other cytotoxic molecules, such as granulysin, which has antimicrobial and proapoptotic effects, and Fas ligand (FasL), which can trigger apoptosis in target cells expressing the Fas receptor. This process plays a crucial role in the innate immune system's ability to eliminate abnormal or infected cells without the need for prior sensitization or recognition by specific antigens [70-72].

In a study conducted by Shapiro et al. [73], a platform for adoptive NK cell transfer was developed to target relapse following haploidentical allogeneic transplant. The treatment demonstrated safety, with the NK cells persisting for several months after administration and infiltrating disease sites including bone marrow and extramedullary environment. These findings suggest that NK cells have the potential to enhance the success of transplantation by decreasing relapse rates and minimizing the occurrence of GVHD. Another significant finding from the study is that the expression of NK inhibitory ligands may provide a possible mechanism of relapse after NK cell adoptive therapy.

In the context of targeting cancer cells, the balance between inhibitory and activatory signals in NK cells becomes particularly relevant. NK cells have the ability to recognize and eliminate cancer cells through a process known as tumor immunosurveillance. The inhibitory signals provided by receptors like KIRs and NKG2A normally prevent NK cells from attacking healthy cells; however, cancer cells can exploit these inhibitory mechanisms by down-regulating MHC-I molecules, thereby evading detection by inhibitory receptors and escaping immune surveillance. The expression of inhibitory receptors such as KIR2DL1, KIR2DL3, KIR3DL1, and NKG2A, along with their corresponding MHC-I ligands, is of significant importance, as they are involved in the education of NK cells to recognize self and nonself MHC. The absence of interaction between inhibitory KIR and their ligands can lead to a hyperresponsiveness state in NK cells resembling the state observed in senescent or exhausted cells [35,74-77]

To counteract this evasion strategy, activatory receptors on NK cells, such as NKG2D and NKp46, play a critical role in recognizing stress-induced ligands or tumor-specific antigens displayed by cancer cells. These activatory signals trigger NK cell activation and cytotoxicity, leading to the destruction of cancer cells. Achieving an optimal balance between inhibitory and activatory signals is essential for effective NK cell-based cancer immunotherapy [78].

An important advantage of K562.Clone1 is its capability to facilitate the expansion and activation of NK cells while conserving the inhibitory receptors. Notably, our results demonstrate that coculturing NK cells with K562.Clone1 cells resulted in a substantial increase in the NK cell population accompanied by elevated expression of activatory receptors, including NKG2D, NKp30, and NKp44, indicating a heightened level of activation and cytotoxic potential against cancer cells [65,67,79].

Additionally, K562.Clone1 cells have the remarkable ability to up-regulate the expression of NKG2D receptor. This receptor interacts with specific ligands, such as MIC-A, MIC-B, and ULBP-1, -2, and -3, which are commonly elevated in various types of cancer, including melanoma, leukemia, myeloma, glioma, prostate, breast, lung, and colon cancers [80-82]. The up-regulation of NKG2D ligands on cancer cells enhances their vulnerability to recognition and elimination by NK cells. When NKG2D receptors on NK cells engage with these ligands on cancer cells, this triggers an activating response within the NK cells themselves. This activation leads to the production of cytokines and the release of cytotoxic factors such as perforin, INF-γ, and TNF-α, ultimately resulting in the efficient elimination of tumor cells [3,83,84]. The ability of K562.Clone1 cells to induce the up-regulation of NKG2D ligands on cancer cells is a significant advantage in NK cell-based cancer immunotherapy by enhancing the susceptibility of cancer cells to NK cell recognition and subsequent elimination [78].

Another important point to consider when constructing an NK cell platform expansion based on feeder cells is the preservation of cytotoxic potency and function. Cytotoxic data obtained from the CAM assay supports the effectiveness of

NK cells expanded ex vivo with K562.Clone1 in killing in vitro U86-MG, glioblastoma lineage. Our results are consistent with previous studies reported by Kmiecik et al. [85] and Golán et al. [86] that demonstrated the strong potential of NK cells to kill U86-MG. This raises the possibility of using NK cells to treat brain cancer, given their extensive interactions with the central nervous system and ability to act as both regulators and mediators of neuroinflammation.

The use of NK cells in the treatment of hematologic malignancies holds great promise and has the potential to yield significant advancements in the field of adoptive NK cell therapy. Numerous clinical trials are currently underway to assess the effectiveness of NK cell therapy in leukemia patients who have experienced relapse or have proven resistant to standard treatments [12,53,60,87]. These trials involve the administration of ex vivo expanded NK cells or genetically modified CAR-NK cells, which are engineered to selectively target and eliminate cancer cells. The objective is to augment the patient's immune response and enhance treatment outcomes. CAR-NK cells have already undergone phase 1/2 testing for the treatment of B cell malignancies and have demonstrated a favorable response in patients. Ongoing research is focused on improving the efficacy, durability, and safety of CAR-NK cells [88].

Preliminary results from these trials have shown encouraging outcomes, with some patients achieving complete remission or experiencing improved disease control. However, it is crucial to acknowledge that these trials are in progress, and further investigation is needed to ascertain the long-term effectiveness and safety of NK cell therapy in hematologic malignancies [88,89].

Recently, cytotoxic assays using 3-dimensional (3D) cultures have been investigated. One specifically—CAR-NK-92 cells—are used in these models to target and bind to a protein known as HER1, which is frequently found in significant quantities on the surface of head and neck carcinomas. The introduction of CAR-NK-92 cells to these 3D cancer cell spheroids resulted in a rapid reduction in the number of cancer cells within a 12-hour time frame, suggesting that the engineered NK cells could effectively infiltrate complex structures such as spheroids, underscoring the importance of predicting their effectiveness more accurately in actual tumors [72,90].

Organ-on-chip (OOC) technology has emerged as a valuable tool in the field of immunotherapy, enabling the replication of complex and dynamic interactions that occur within the tumor microenvironment. One important feature that OOC models have revealed is the ability of NK cells to initiate a process known as extravasation toward physically separated tumor areas. This process involves the migration of NK cells under physiologic fluid flow and infiltration within a 3D tumor matrix, and confirms that NK cells can migrate upward following an active, chemoattractant-driven extravasation without being dependent on fluid flow. This finding highlights the ability of NK cells to navigate complex structures and engage in tumor cell recognition and elimination [91].

Brazil has seen a growing number of initiatives focused on immunotherapy, particularly in the areas of CAR-T and NK cell therapy. The National Cellular Therapy Network (RNTC) consists of cellular technology centers and laboratories that have been carefully selected by the Department of Science and Technology of the Ministry of Health. The main objective of the RNTC is to foster collaboration among researchers throughout the country, facilitating the exchange of knowledge and advancements in therapeutic approaches. Collaboration with international organizations, such as the International Society for Cell and Gene Therapy and the International Society for Stem Cell Research, helps disseminate knowledge in the field of cell and gene therapy. The RNTC also supports the development of good manufacturing practices (GMP) locally, which is crucial for ensuring the high-quality production of cell therapy products [92,93]. As part of our ongoing efforts to enhance the manufacturing aspect of these therapies, we propose the establishment of a feeder cell on national territory, with a primary focus on facilitating NK cell immunotherapy in Brazil [94].

As mentioned earlier, feeder cells play a critical role in the ex vivo expansion of NK cells. Constructing feeder cells is laborious, and thus they are often protected by patents held by the centers that developed them. K562.Clone1 feeder cells have shown great potential as a nationally available feeder system. Through cocultivation with peripheral blood NK cells, there is great potential to expand NK cells and conserve their principal phenotypic characteristics, well-balanced in the expression of activatory and inhibitory receptors, that are crucial for a precise antitumor response. Coculture of NK cells with K562.Clone1 feeder cells enhances the cytotoxic potency of granules and receptors, such as FASL.

We strongly believe that providing local access to NK cell therapy platforms will expedite Brazilian patients' access to this advanced therapy and

also significantly contribute to advances in cancer treatment throughout the nation.

CONCLUSION

Our group has successfully constructed a readily available national feeder cell, K562.Clone1. By coculturing peripheral NK cells with K562.Clone1 feeder cells, we have achieved a remarkable expansion of up to 100-fold. Importantly, this coculture system has demonstrated the ability to sustain the activation and functionality of NK cells, preserving their essential functions, such as recognizing missing self and exerting natural cytotoxicity against tumor cells. The development of this feeder cell platform provides Brazilian researchers with a valuable tool for the ex vivo expansion of NK cells. It paves the way for future advancements in therapeutic approaches, offering new possibilities for targeted immunotherapy and potentially improving patient outcomes.

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