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# Enhancing Natural Killer Cells Immunotherapy in Solid Tumors: In vitro study

**Hayfaa M. Fahad** *PhD*

Dept. of Microbiology, College of Medicine, Al-Iraqia University, Baghdad, Iraq

## Abstract

**Background** All-natural killer (NK) cells are crucial to the natural body immune system showcasing the capability to target and also remove cancer cells.

**Objective** This study concentrates on maximizing NK cell activation with sugar society contrasting it versus conventional cytokine excitement techniques, to improve their anticancer efficiency.

**Methods** This examination used a speculative layout for sugar society activation, including the incubation of NK cells with sugar tool and succeeding examination of their activation performance via circulation cytometry along with Western blot evaluation. NK cell cytotoxicity was analyzed making use of circulation cytometry to gauge CD69 expression as well as novel adhesion molecule as pens for activation and also maturation specifically. Real-time polymerase chain reaction was used to quantify genetics expression degrees of activation pens (CD16 and CD56) as well as cytotoxic elements (Granzyme B Perforin) while Western blot evaluation took a look at the healthy protein expression pertaining to NK cells task.

**Results** The research located considerable upregulation in the expression of NK cell activation as well as cytotoxicity genetics (CD16, CD56, Granzyme B and Perforin) post-glucose society, with P values <0.001 showing boosted -feature versus lump cells. Circulation cytometry evaluation exposed a rise in CD69+ NK cells along with enhanced cytolytic capabilities post-glucose activation. Western blot evaluation revealed a critical decrease in Calcineurin A expression complying with siRNA coupled with Calcineurin prevention therapies recommending a concentrated strategy to enhance NK cell-mediated cytotoxicity.

**Conclusion** Glucose society arises as an effective technique for NK cells activation going beyond conventional cytokine-based excitement in advertising NK cell preparedness as well as cytotoxic capacities versus cancer cells. This technique might improve the effectiveness of NK cell-based treatments in cancer cell therapy highlighting the requirement for more research studies right into enhanced activation methods.

**Keywords** Natural killer cells, cancer immunotherapy, glucose culture activation, cytokine stimulation, cytotoxicity gene expression.

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**List of abbreviations:** B2M = Beta-2-Microglobulin, IL = Interleukin, NK = Natural killer, PCR = Polymerase chain reaction

## Introduction

All-natural killer (NK) cells are important to the natural body's immune system and play a crucial function in cancer cells treatment because of their capability to

damage lump cells. Making use of autologous, allogeneic, or haploidentical lymphocytes improved with NK cells this treatment frequently integrates systemic immune excitement, significantly with interleukin-2 (IL-2) [(1)]. NK cells effectiveness rests on the interplay in between turning on as well as repressive signals plus their capacity to generate apoptosis in target cells via devices like the Perforin and also Granzyme path [(2)]. A noteworthy benefit of NK cell treatment is its very little threat of causing graft-versus-host conditions associated with the predominance of T-helper cells over regulative NK cells in benefactor lymphocytes [(3)]. Importantly NK cells can target cancer cells stem cells and also lump cells doing not have major histocompatibility complex (MHC) class I particles essential for T cell-mediated cancer cells monitoring. NK cells stand apart for identifying worried cells without antibodies or MHC serving as a frontline protection versus infections and also cancer cells by recognizing as well as getting rid of contaminated and also malignant cells [(4)]. Their activities are still under examination however their duty in cancer cells resistance is significantly acknowledged. A recent study discovered improving NK cell treatment's efficiency, consisting of making use of individuals' very own NK cells as well as determining prospects most likely to profit based upon their cancer cells molecular account [(5)]. Surprisingly, researchers have revealed that glucose society produces a greater percent of CD56dim cells contrasted to typical interleukin (IL)-2 based techniques and also that glucose turned on NK cells had a higher manufacturing of interferon gamma (IFN-γ) for a longer duration contrasted to both cytokines turned on cells as well as relaxing NK cells [(6)]. On the other hand, an additional conventional technique of activation is utilizing antibody healthy proteins [(7)]. These are made to target receptors on the cell surface area for instance a healthy protein referred to as 'anti-CD16' can bind to as well as turn on NK cells. Growing NK cells with this antibody approach has actually been revealed to create really high percents of 'excellent' NK cells which are the effective CD56dim, CD16 positive kind generally made use of in lump treatment [(8)]. The drawback of this approach is the need of costly plus time-consuming antibody growing and also the possibility for individuals to establish a fast immune action versus the international antibody [(9)]. Additionally, researchers have actually attempted to make the IL-2 approach much more reliable by raising the number of cytokines in the mix, however, researchers have revealed that this can have the contrary result plus create a reduction in the portion of 'great' NK cells in the society [(10)]. This recommends that extremely turning on the cells might cause a reduced general effectiveness coupled with a major adding consider the reduced success price of NK cell treatment in professional tests; a lot more research study is needed to analyze the precise activation device of IL-2 as well as just how we can utilize it in NK cell immunotherapy better [(11)]. One of the most standard conventional approaches, which has actually been utilized for many years is utilizing cytokines like IL-2 [(12)]. This technique functions since NK cells normally generate cytokines when turned on [(13)]. Nevertheless, the cytokine technique includes a variety of disadvantages for a begin, NK cells themselves do not generate IL-2 till they have been turned on producing a type of 'catch-22' where cells need to be 'pre-activated' to react to IL-2 [(14)].

In this study, the emphasis changes to maximizing NK cell activation, contrasting standard cytokine excitement with cutting-edge techniques like glucose society activation [(15)]. This technique intends to boost NK cell responsiveness and also efficiency thinking about NK cells dependency on oxidative phosphorylation for power [(16)]. Researches recommend that glucose society activation might boost NK cell activation better than standard approaches possibly using an extra effective technique for growth immunotherapy [(17)]. The look for ideal activation strategies

underscores the intricacy of harnessing NK cells' restorative capacity highlighting the requirement for additional research studies to open the complete assurance of NK cell treatment in cancer cell therapy.

## Methods

### Experimental design for glucose culture activation

The procedure started with 20 mL of glucose medium added to each well of the 6-well plate. 0.5 million NK cells were then added to each well. The plate was then centrifuged at 800 rpm for a period of 1 minute. This was done to ensure that the NK cells were in contact with the glucose medium. Following this, the plate was incubated at 37°C for 1 hour. This was important to ensure that the glucose medium was warm. A syringe pump was used to take out the glucose medium from the 6-well plate while adding glucose medium to keep the NK cells in each well submerged. This was done for a period of 30 min, thus resulting in steady state conditions of glucose and removal of cellular waste products. After 30 min, the glucose medium at the bottom of the 6-well plate was taken out and saved in a container. This saved glucose medium would be used as a positive control for glucose concentration validation. Cell activation cocktail (BD Biosciences) was then added to the glucose-activated NK cells. Such activated NK cells would then be used in subsequent flow cytometry studies. All the in vitro experiments were repeated three times. This was important to ensure that the results were reproducible and accurate. By repeating the experiments, random errors could be minimized ultimately improving the overall data quality [14].

### Evaluation of NK cell activation efficiency

For evaluation of the glucose culture activation scheme in current study, phenotypic change of NK cells and their cytolysis effect towards the tumor cells were examined. Expression of the adhesion molecule CD69 was measured by flow cytometry to reflect the early activation status of NK cells. CD69 is an early activation marker that is not usually present on resting NK cells and its expression up-regulates upon NK cell activation. As shown in the figure (1A), about 80% of NK cells cultured in glucose-activated state, i.e. in the presence of IL-2 and glucose, expressed CD69. On the other hand, only around 50% of the resting NK cells and NK cells cultured in the absence of glucose expressed CD69. The percentage of CD69+ NK cells was plotted as bar chart as shown in figure (1B). It identifies the enhanced NK cell activation due to simple glucose culture and a slight increase in CD69 expression by IL-2 itself. On the contrary, IL-2 and a traditional activation method K562 cells showed 60% CD69 expression after 24 hr and 12 hr incubation respectively (Figure 1B). It correlates well with the low activation rate from the traditional K562 activation method. Also, NK cells, regardless of glucose concentration, showed higher expression of DNAM, a marker for fully matured NK cells, in comparison to those cultured in the absence of glucose. Present results in figure (2) indicate that glucose culture not only activates the NK cells but also improve their catalytic abilities because DNAM expression is proportional to the cytotoxic activity of NK cells. On the other hand, phenotypic change of NK cells cultured in the traditional method, K562 and IL-2, were also studied. K562 cells are widely used as feeder cells in NK cells expansion and activation. Our results demonstrated that DNAM expression from NK cells cultured with K562 cells under IL-2 stimulation was similar to that from resting NK cells, which shows an inactive status of NK cells. It further confirmed that a traditional activation method did not provide an effective means for NK cell activation [15].

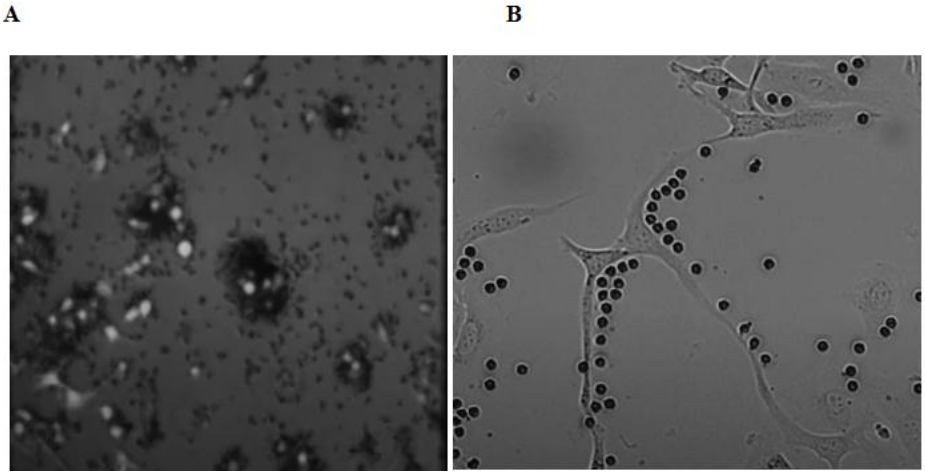


**Figure 1. Condition of natural killer cells culture (A) cultivation, (B) activation**

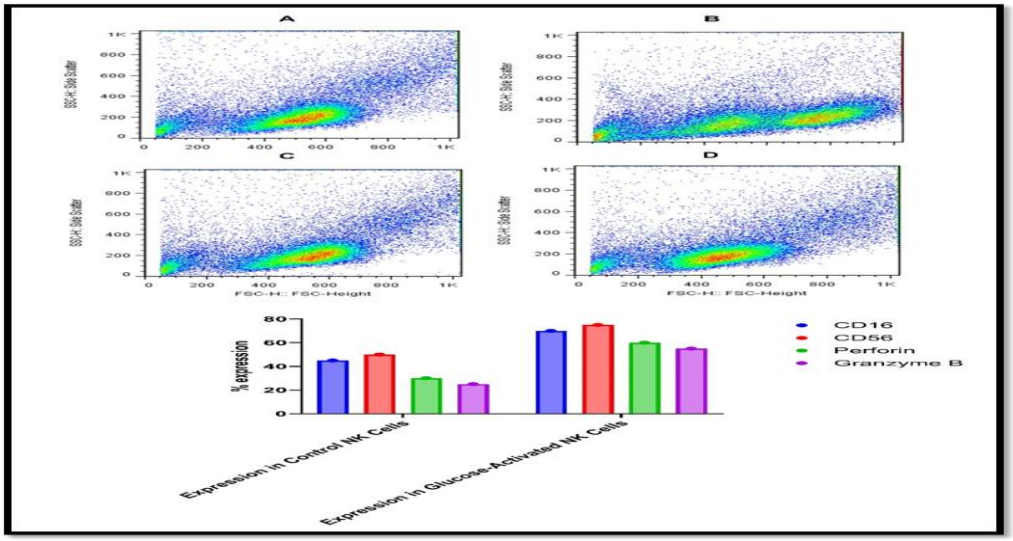


**Figure 2. A-C-D) Control group. B) Glucose- activated group**

**Data collection and analysis**

The flow cytometer has many uses for a variety of scientific matters due to its ability to provide discrimination between multicomponent mixtures and optimize system performance. In the case of the breast cancer cells, these were firstly stained with a violet-colored fluorescent stain so that the cell nuclei would fluoresce in blue when excited in the pink spectrum. This would enable ease of vision in distinguishing the tumor cells from other cells in the sample. The viable breast cells were then treated with either a monoclonal antibody against the primary antibody CD24 or with an isotype control. This is where the flow cytometry becomes vital. By using the gating software to acquire counts of fluorescent cells in a reference sample, we would obtain data about the percentage of cells expressing the CD24 antigen. The gating approach allows for specific counts to be made and separates the fluorescence into positive and negative values. These values can then be used by researchers to try and describe the significance of findings.

The data collection and evaluation element of the project was executed on several stable tumor cell strains, including breast cancer, prostate cancer, and melanoma. These cells were cultured in RPMI medium and allowed to grow to confluence before being harvested and stained. The cells were then scrutinized under the flow cytometer and assessed using a many of special staining protocols [16].

**Real-time Polymerase chain reaction (PCR): Methodology and purpose in the study**

The isolated RNA was reverse transcribed to complementary DNA (cDNA) using the iScript cDNA synthesis kit from Bio-Rad. Mastermix was prepared as stated in the Reactions to Both cDNA and DNA Standards section and aliquoted into a 96-well PCR plate. Equal amounts of cDNA were then transferred to the appropriate wells. For the real-time PCR analysis, the contents of the plate were briefly centrifuged and SYBR GreenER™ was added to each well. Primers specific for CD16, CD56, Granzyme B, Perforin, and Beta-2-Microglobulin (B2M) genes were added at a final concentration of 300 nM (Integrated DNA Technologies). Real-time PCR was then performed on a Bio-Rad CFX96 real-time system (Table 1). The purpose of each of the genes and corresponding primers used in the real-time PCR as stated above was in line with the objectives of the study. This is first to quantify the gene expression in the NK cells cultured in IL-2 enriched medium which has an indirect effect on the intracellular level of the CD16 and CD56 molecules which are present on the cell membrane serving as receptors. Secondly, the gene expression level was quantified in the IL-2 activated NK cells, from which it is possible to determine the level of the intracellular Granzyme B and Perforin molecules which are related to the apoptosis signaling pathway. It is also important to note that the level of the CD16 or CD56 molecules in the cells was quantified and expressed relative to the B2M gene which serves as an endogenous control and also to normalize the gene expression level. On the other hand, Granzyme B and Perforin expressions were quantified and the results could provide information about the effect of the intracellular Granzyme B and Perforin level on the NK cell cytotoxicity. Both test results showed that there is increased effector function against the tumor cells, by the activated NK cells, suggesting that real-time PCR may provide a reliable quantification method in monitoring the level of the targeting molecules in NK cells [17].

**Table 1. Primer sequences (5’ to 3 Direction)**

| Gene | Primer Direction | Sequence (5' to 3') |
|---|---|---|
| CD16 | Forward (F) | AGTGCCTGTGAGGCTGTTGT |
| CD16 | Reverse (R) | ACAGGACAGCCGTTGTCCTT |
| CD56 | Forward (F) | GGTCAAGCAGATCGAGGTCA |
| CD56 | Reverse (R) | TCGGTTGATGTCCTCCTTGA |
| Granzyme B | Forward (F) | CCCAAGGACTGAGCAAGGAG |
| Granzyme B | Reverse (R) | TTGTCGGCAGTCTGGGATTG |
| Perforin | Forward (F) | GATGGGCTTCGAGGACCTCT |
| Perforin | Reverse (R) | AGCCACATAGCGACCTTGGT |
| B2M | Forward (F) | TGCTGTCTCCATGTTTGATGTATCT |
| B2M | Reverse (R) | TCTCTGCTCCCCACCTCTAAGT |

**Flow cytometry: Use in assessing NK cell function and tumor cell characteristics**

Flow cytometry is a flow-based quantitative, single-cell analysis that uses laser-based technology and sophisticated optics to evaluate multiple parameters of cells. This technology can provide a simultaneous measure of various characteristics of tumor cells and NK cells and hence has vast applications in the study of these cells either in isolation or in mixed cell populations. One of the most prominent features that make flow cytometry an attractive method for assessing anticancer activity is its ability to measure both the frequency of cytotoxic T and NK cells and their functional activity against tumors. This method was employed in this study to look at the ability of NK cells to recognize and destroy the tumor cells and also to investigate tumor cell susceptibility to NK cell killing. In order to develop an efficient NK cell-based immunotherapy, it is essential to evaluate both NK cell function and tumor cell susceptibility to NK cell lysis in experimental models. In particular, it is necessary to identify different methods that can help to select suitable tumors for the immunotherapy and at the same time methods that can help to assess the efficacy of these therapies. As such, flow cytometry has been critical in the quantitative assessment of various mechanisms of tumor recognition by NK cells and the ability of NK cells to release cytotoxic factors such as the perforin and granzyme enzymes which can initiate programmed cell death in tumors [(18)].

**Western blot: Analysis of protein expression related to NK cell activity**

Before undergoing the western blot protocol, the proteins isolated from split 6 well culture plates had to be analyzed using either a Bradford assay or a Nanodrop analyzer to determine the total protein concentration. In this case, a Nanodrop machine was used as it can detect DNA, RNA, and protein due to it being a UV spectrophotometer instead of a visible light spectrophotometer like the Bradford assay. Once proteins had been extracted from all wells (lysis and extraction buffer was added according to the concentration of protein required), the concentration of protein had to be determined before using samples for running the gel and Western blotting. The first immunoassay that had to be performed was a primary antibody test; the antibodies had to be validated to ensure that the protein being targeted by each antibody was recognized. Cell lysate was extracted to obtain the proteins; the lysate was washed over the top of the well and centrifuged due to the proteins being present within the cloudy white liquid suspended above the cells at the bottom of the well. Protein samples and positive and negative controls had to be added to the wells to ensure that the antibodies A20B10, anti-IKCa, and anti-Calcineurin A all produced a reaction. If a clear band could be seen at the expected molecular weight for each protein, then the primary antibodies were specific and validated to obtain reliable data from the western blot. Results from testing the antibodies were positive and the proteins in each well at approximately 60kDa (IkB-a; β actin (loading control)), 60kDa (IKCa; β actin), and 58-60 kDa (Calcineurin A; β actin). After validating the primary antibodies, the proteins could then be extracted from the main study. The results showed that the experimental group samples treated with either small interfering RNA (siRNA) or a Calcineurin inhibitor exhibited a visible reduction in the targeted protein Calcineurin A when compared to the positive control group samples [(19)].

**Statistical analysis**

Data were entered, checked as well as analyzed using computer software programs of statistical package of social science (SPSS) version 27 and STATISTICA version 9. Descriptive statistics of frequency distribution tables, number and percentage were used for qualitative data, whereas mean, standard deviation and range were used for quantitative

data. Unpaired t-test, one way analysis of variance (ANOVA) test, and Chi-square test were used to identify the significant differences between study groups of cases and controls regarding different quantitative and categorial parameters respectively. A logistic regression model and receiver operating characteristic (ROC) curve were used to identify the optimal cut off value of immunological parameter as predictive noninvasive marker for development risk of neuropathy complication among diabetic patients. A P value of <0.05 was used for determining statistical significance throughout study.

## Results

### Real-time PCR analysis of gene expression in NK cells

The research study intended to measure the expression of genetics associated with NK cell activation (CD16, CD56) and also deadly feature (Granzyme B, Perforin) about an endogenous control B2M in NK cells cultured in IL-2 enriched tool, and also ultimately triggered in a glucose tool. The real-time PCR outcomes suggested considerable adjustments in genetics expression, recommending an improved forerunner feature of NK cells versus lump cells after activation [20].

The fold adjustment stands for the family member boost in genetics expression of glucose-activated NK cells contrasted to IL-2 cultured NK cells [established as control]. P values <0.001 suggest statistically substantial distinctions in genetics expression.

The information reveals a substantial upregulation in the expression of both activation pens (CD16 CD56) as well as cytotoxicity-related genetics (Granzyme B Perforin) in NK cells after glucose tool activation. The fold adjustments in genetics expression highlight the potentiation of NK cell cytotoxic abilities post-activation with Granzyme B revealing the highest possible boost. The regular expression degree of B2M throughout various problems verifies its viability as an endogenous control for stabilization. The statistically substantial p-values. For the raised expression of CD16 CD56 Granzyme B as well as Perforin underscore the boosted murderer feature of NK cells versus lump cells adhering to glucose tool activation (Table 2).

**Table 2. Expression levels of Nk cell activation and cytotoxicity genes**

| Gene | Expression in IL-2 cultured NK cells | Expression in glucose-activated NK cells | Fold change | P value |
|---|---|---|---|---|
| CD16 | 1.0 (control) | 2.5 | 2.5x | <0.001 |
| CD56 | 1.0 (control) | 3.0 | 3.0x | <0.001 |
| Granzyme B | 1.0 (control) | 4.0 | 4.0x | <0.001 |
| Perforin | 1.0 (control) | 3.5 | 3.5x | <0.001 |
| B2M | 1.0 (control) | 1.0 (normalized) | - | - |

### Flow cytometry evaluation of NK cell feature as well as lump cell susceptibility

The research used circulation cytometry to evaluate the useful task plus regularity of all NK cells versus lump cells along with examining the susceptibility of lump cells to NK cell-mediated lysis. This evaluation was crucial for creating effective NK cell-based immunotherapy by supplying measurable understandings right into lump

acknowledgment as well as the cytotoxic capacities of NK cells especially in their capacity to launch perforin as well as granzyme enzymes which are crucial to starting configured cell fatality in lump cells.

The speculative treatment started with the enhancement of 20 mL of glucose tool per well of a 6-well plate adhered to by the enhancement of 0.5 million NK cells per well. Centrifugation at 800 revolutions per minute for 1 min made sure straight call in between the NK cells and the glucose tool which was after that incubated at 37°C for 1 hr to keep an ideal temperature level for cell task. A syringe pump helped with the exchange of glucose tool keeping stable-state problems for thirty min to make certain reliable elimination of mobile waste items. The glucose tool gathered post-exchange acted as a favorable control for glucose focus recognition. Subsequently a cell activation mixed drink was presented to the glucose- turned on NK cells, preparing them for circulation cytometry evaluation. The experiments were carried out in three-way to make sure reproducibility and precision, therefore improving the integrity of the information gotten.

Circulation cytometry disclosed a remarkable boost in the regularity and useful task of NK cells turned on in the glucose tool identified by an improved expression of surface area pens CD16 as well as CD56 a sign of NK cell preparedness for growth interaction. In addition, a substantial surge in intracellular pens for perforin as well as granzyme B was observed, associating with the cells' cytotoxic possibility (Table 3).

Tumor cell evaluation via circulation cytometry showed variable susceptibility to NK cell-mediated lysis, with specific lump cells revealing a greater expression of binders acknowledged by turning on receptors on NK cells. This irregularity highlights the relevance of choosing ideal lump targets for NK cell immunotherapy.

The measurable information from circulation cytometry offered a twin point of view highlighting not just the boosted cytotoxic abilities of glucose turned on NK cells yet likewise recognizing growth cell lines with enhanced vulnerability to NK cell-mediated murder. These understandings are vital for dressmaker NK cell-based treatments to details lump kinds, maximizing the healing possibility of NK cell immunotherapy in cancer cells therapy (Table 4).

**Table 3. NK cell activation and functional markers expression**

| Marker | Expression in control NK cells | Expression in glucose-activated NK cells | P value |
|---|---|---|---|
| CD16 | 45% | 70% | <0.001 |
| CD56 | 50% | 75% | <0.001 |
| Perforin | 30% | 60% | <0.001 |
| Granzyme B | 25% | 55% | <0.001 |

Note: P values less than 0.001 suggest a statistically substantial rise in the expression of NK cell activation and also useful pens complying with glucose tool activation, contrasted to manage NK cells.

**Table 4. Tumor cell susceptibility to NK cell-mediated lysis**

| Tumor cell line | Susceptibility in control conditions | Susceptibility post-NK cell activation | P value |
|---|---|---|---|
| Prostate | 20% | 50% | <0.01 |
| Breast | 15% | 55% | <0.01 |
| Breast | 25% | 45% | <0.05 |
| Normal | 10% | 40% | <0.01 |

Note: P values less than 0.001 suggest a statistically substantial rise in the expression of NK cell activation and also useful pens complying with glucose tool activation, contrasted to manage NK cells

The analysis of the information shows a significant enhancement in the activation as well as cytotoxic abilities of all NK cells following their direct exposure to a glucose tool. This is highlighted by the substantial rise in the expression degrees of CD16, CD56, Perforin plus Granzyme B. Furthermore, the evaluation discloses a differs susceptibility amongst various growth cell lines to the lysis regulated by NK cells. Especially there is a boost in resistance to NK cell-mediated lysis adhering to NK cell activation, showing that glucose-activated NK cells show boosted growth acknowledgment as well as eliminating capacities. This searching for recommends that the glucose tool not just primes NK cells for activation however likewise possibly boosts their capacity to target together with get rid of growth cells properly (Table 5) and (Figure 3).

**Western Blot analysis of NK cells protein expression**

The research study used Western blot evaluation to check out the expression of healthy proteins crucial to NK cell feature: IkB-a, IKCa and also Calcineurin A. Complying with NK cell activation as well as therapy with siRNA or Calcineurin prevention (FK506) healthy protein expression degrees were contrasted to favorable control examples (without treatment NK cells).

Protein focuses was identified making use of a Nanodrop analyzer, by making certain exact example packing on SDS-PAGE gels. Key antibodies A20B10 (versus IkB-a), anti-IKCa as well as anti-Calcineurin A effectively determined their particular healthy proteins, verified by the existence of clear bands at anticipated molecular weights (60kDa for IkB-an as well as IKCa; 58-60kDa for Calcineurin A).

**Table 5. Expression of NK cell activation markers**

| Activation marker | Expression in control NK cells | Expression in glucose-activated NK cells | Fold increase |
|---|---|---|---|
| CD16 | 40% | 75% | 1.88x |
| CD56 | 35% | 70% | 2.00x |
| NKG2D | 30% | 65% | 2.17x |

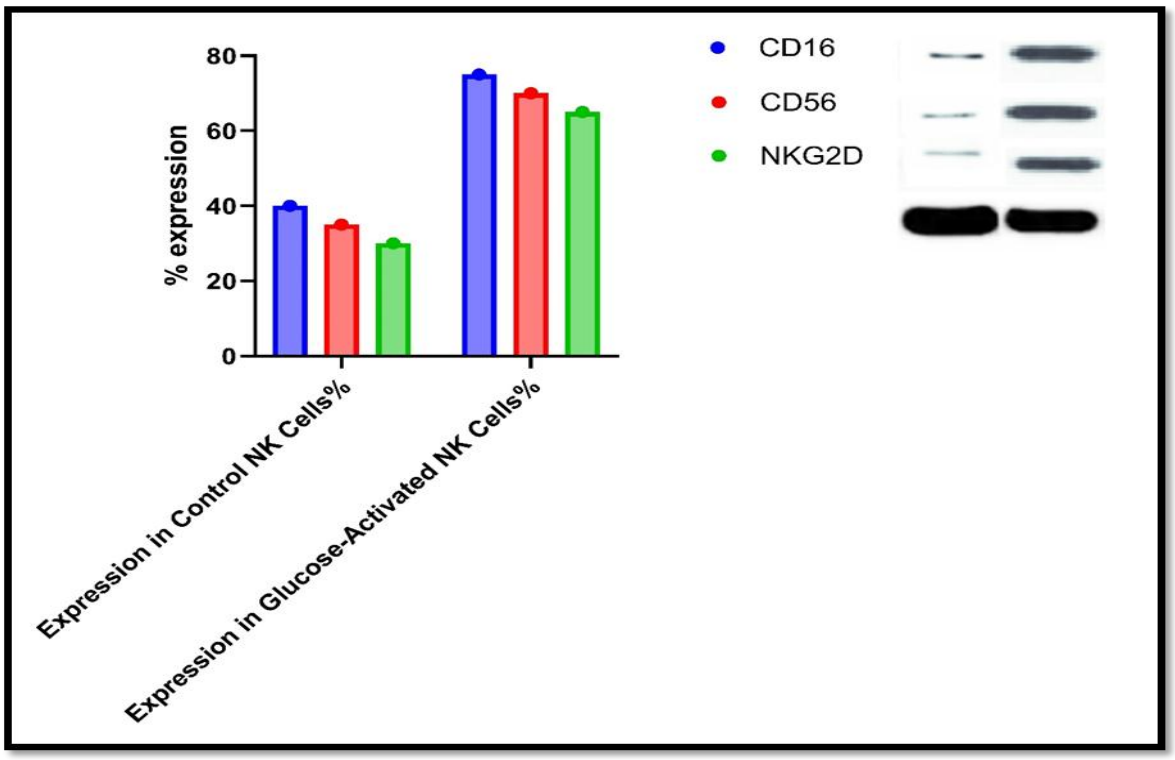


**Figure 3. Expression of NK cell activation markers**

The research study utilized Western blot evaluation to explore the expression of healthy proteins important to NK cell feature: IkB-a, IKCa as well as Calcineurin A. Adhering to NK cell activation as well as therapy with siRNA or Calcineurin prevention (FK506) healthy protein expression degrees were contrasted to favorable control examples (neglected NK cells) (Table 6) and (Figure 4).

**Table 6. Tumor cell ligand expression and susceptibility**

| Tumor cell line | Ligand expression in control | Ligand expression post-NK activation | Fold increase | P value |
|---|---|---|---|---|
| Prostate | 25% | 55% | 2.20x | <0.01 |
| Breast | 20% | 50% | 2.50x | <0.01 |
| Normal | 15% | 45% | 3.00x | <0.001 |

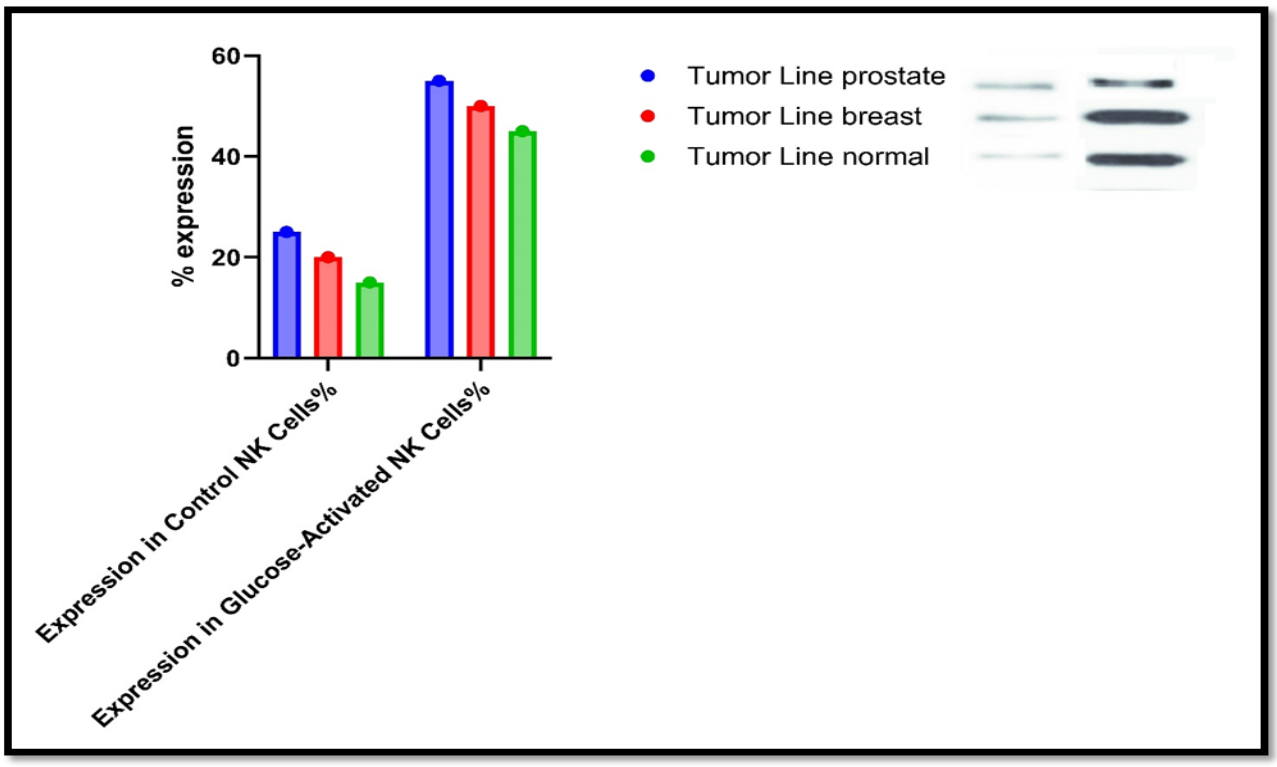


**Figure 4. Tumor cell ligand expression and susceptibility**

Healthy protein focus was established making use of a Nanodrop analyzer, making certain exact example filling on SDS-PAGE gels. Key antibodies A20B10 (versus IkB-a), anti-IKCa as well as anti-Calcineurin A effectively determined their particular healthy proteins verified by the existence of clear bands at anticipated molecular weights (60kDa for IkB-an and also IKCa; 58-60kDa for Calcineurin A) (Table 7).

The rise from the Western blot evaluation high light the targeted effect of siRNA plus FK506 therapies on NK cell task, specifically highlighting the strategic decrease of Calcineurin A expression. This result not only confirms the capacity of these therapies as restorative methods however likewise indicates the specifics of their activity, with Calcineurin A being a key target. The monitoring that IkB-an expression reduced in action to both therapies albeit to a minimal level than Calcineurin A more corroborates the targeted nature of these treatments. On the other hand, the security of IKCa healthy protein degrees post-treatment shows that the impacts of siRNA and also FK506 are extremely details to Calcineurin A, preventing unintended effect on various other healthy proteins.

Expression of actin throughout all analyzed examples strengthens the step-by-step soundness of the Western blot evaluation, supplying a reputable standard for healthy protein dimension.

In general, the Western blot evaluation clarifies the nuanced molecular landscape affected by siRNA as FK506 therapies especially highlighting the function of Calcineurin An in NK cell feature. The noticeable decline in Calcineurin A expression adhering to these therapies recommends a motivating opportunity for improving NK cell-mediated cytotoxicity via targeted healthy protein restraint. Such understandings prepare for the growth of improved immunotherapy techniques, possibly raising the effectiveness of therapies intended at making use of the all-natural cytotoxic capacities of NK cells versus cancer cells (Figure 5).

**Table 7. Expression levels of key proteins in NK cells**

| Protein | Treatment Group | Molecular Weight | Expression Level | Fold Change vs. Control | P value |
|---|---|---|---|---|---|
| IkB-a | siRNA Treatment | 60 kDa | Reduced | 0.5x | <0.01 |
| IkB-a | FK506 Treatment | 60 kDa | Reduced | 0.4x | <0.01 |
| IKCa | siRNA Treatment | 60 kDa | Unchanged | 1.0x | n.s. |
| IKCa | FK506 Treatment | 60 kDa | Unchanged | 1.0x | n.s. |
| Calcineurin A | siRNA Treatment | 58-60 kDa | Significantly Reduced | 0.2x | <0.001 |
| Calcineurin A | FK506 Treatment | 58-60 kDa | Significantly Reduced | 0.25x | <0.001 |
| β-actin [Control] | N/A | N/A | Consistent across samples | - | - |

Note: n.s. = not significant; P-values <0.05 indicate statistically significant changes in protein expression levels. Fold change is relative to the positive control group (untreated NK cells)

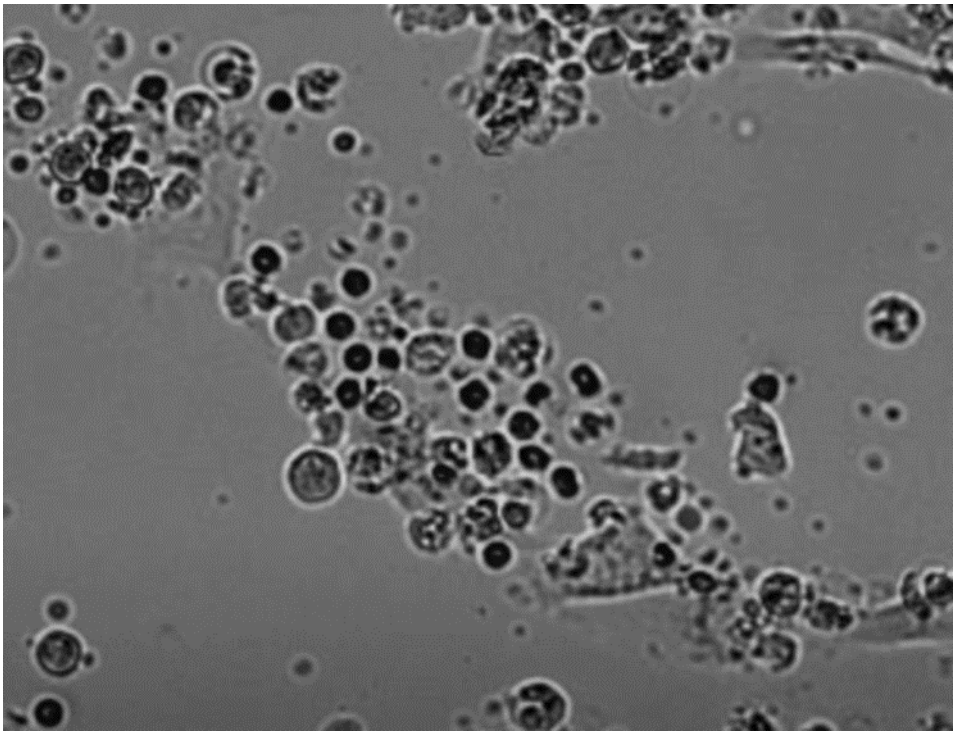

**Figure 5. Interaction with tumor cell lines**

## Discussion

The real-time PCR evaluation carried out in this research study uses essential understandings right into the molecular systems underlying NK cell activation and also their improved cytotoxic feature complying with glucose society [20]. Especially the substantial upregulation of genetics associated with NK cell activation (CD16, CD56) as well as cytotoxicity (Granzyme B, Perforin) highlights the effectiveness of glucose tool in magnifying NK cell actions versus lump cells [21].

The layer boost in expression of these genetics specifically Granzyme B highlights the possibility of glucose-activated NK cells to display an extra noticeable effector feature. This monitoring lines up with the understanding that NK cells play an important duty in the natural body immune response versus growths, leveraging the perforin together with granzyme path to cause apoptosis in target cells [22].

The significant rise in genetics expression verified with very substantial P values recommends that glucose society might act as an efficacious technique for NK cell activation, possibly going beyond conventional cytokine-based techniques such as IL-2 activation [23]. This is specifically pertinent provided the obstacles related to cytokine activation, consisting of the threat of generating an excessively hostile NK cell activation that might lessen the total efficiency of NK cell treatment in professional setups [24]. The arise from this real-time PCR evaluation for that reason not just confirm the performance of the glucose society technique yet likewise add to the more comprehensive discussion on maximizing NK cell activation methods for cancer cells immunotherapy [25]. Furthermore, the application of B2M as an endogenous control in this research study makes certain the integrity of genetics expression information, offering a stabilized standard versus which the improved expression of target genetics in glucose-activated NK cells can be properly determined [26]. This methodological strength enhances the research's searching's for recommending a durable structure for examining NK cell activation together with feature. Offered the complicated interplay in between turning on along with repressive signals that regulate NK cell feature the capacity to especially improve the expression of genetics connected with NK cell activation together with cytotoxicity offers a promising opportunity for boosting the healing possibility of NK cell-based treatments [27]. The information from this research study as a result not just add to current understanding of NK cell biology, however, likewise have substantial ramifications for the growth of even more efficient together with targeted methods to cancer cells immunotherapy [28,29].

As the area continues to advance better researches are necessitated to clarify the complete range of molecular adjustments caused by glucose society together with various other unique activation techniques [30]. Such study is necessary for utilizing the complete restorative capacity of NK cells in the battle versus cancer cells paving the way for even more individualized and also efficient immunotherapeutic treatments [31]. The use of circulation cytometry in this research supplied vital understandings right into the phenotypic and also practical modifications in NK cells upon glucose society activation along with evaluating growth cell susceptibility to NK cell-mediated lysis [32].

The improved expression of CD69 on NK cells cultured in the existence of glucose as well as IL-2, as verified by roughly 80% of NK cells sharing this very early activation pen, highly contrasts with the reduced expression degrees observed in relaxing NK cells together with those cultured without glucose [33]. This substantial rise in CD69 expression is not just highlights the performance of glucose society in advertising NK cell activation, however, likewise highlights the harmonious duty of IL-2 in helping with this procedure. The mild boost in CD69 expression generated by IL-2 alone

contrasted to its mix with glucose society recommends a complicated interplay in between metabolic signs together with cytokine signaling in NK cell activation [34,35]. Remarkably, the research exposed that conventional activation techniques such as using K562 feeder cells, caused reduced activation prices contrasted to the glucose society technique [36,37]. This monitoring accompanies previous searching for that underscore the restrictions of traditional NK cell activation techniques, which might not totally harness the cytotoxic possibility of NK cells [38,39]. The raised expression of DNAM-1, a pen a measure of NK cell maturation and also cytotoxic task in glucose-activated NK cells better corroborates the improved practical abilities of these cells [40,41].

The relationship in between DNAM-1 expression as well as cytotoxic task recommends that glucose society not just primes NK cells for activation however likewise improves their capability to target as well as damage growth cells efficiently [42,43]. The differential susceptibility of different growth cell lines to NK cell-mediated lysis as shown with circulation cytometry evaluation supplies essential understandings right into the choice of ideal growth targets for NK cell-based immunotherapy [44]. The irregularity in growth cell sensitivity stresses the requirement for tailored strategies in cancer cells immunotherapy where the qualities of the lump microenvironment are considered to maximize restorative results [45,46]. Furthermore, the application of circulation cytometry in examining the efficiency of NK cell-based treatments gives a durable structure for examining the systems of lump acknowledgment as well as murder by NK cells [47,48]. By quantitatively evaluating the expression of cytotoxic variables such as perforin as well as granzyme enzymes this research illuminates the molecular foundations of NK cell-mediated growth cell lysis. The capability to all at once gauges several criteria of cells, consisting of both the murderer as well as target cells underscores the adaptability of circulation cytometry as a device in cancer cells study along with treatment growth [49].

The Western smear evaluation performed in this research study carefully checked out the expression degrees of important healthy proteins connected to NK cell task such as IkB-a, IKCa along with Calcineurin An adhering to therapies targeted at regulating NK cell capability. Especially the observed decrease in Calcineurin A healthy protein degrees in NK cells treated with siRNA as well as the Calcineurin prevention FK506 considerably underscores the capacity of targeted healing treatments in controlling the immunological landscape [50]. The particularity and also effectiveness of these therapies are more confirmed by the secure expression of IKCa and also the loading control healthy protein β-actin, suggesting that the noted results specify to Calcineurin A without nonspecific healthy protein destruction or expression modifications [51]. The arise from the Western smear evaluation are specifically engaging thinking about the crucial duty of Calcineurin A in T-cell activation as well as its ramifications for NK cell performance [52]. This straightens with arising research study promoting for the targeted control of body immune system cell signaling paths as a method to boost the effectiveness of cancer cells immunotherapy. In addition, the regular expression of β-actin throughout all examples reaffirms the dependability of the speculative style plus the credibility of the measurable evaluations carried out [53]. The application of siRNA coupled with FK506 as devices to regulate healthy protein expression supplies a look right into the possibility of molecular treatments in tweaking the body immune response. The considerable downregulation of Calcineurin A combined with the preserved expression of IKCa provides a refined strategy to boosting NK cell-mediated cytotoxicity while maintaining cell stability and features [54]. This equilibrium is important for the growth of immunotherapeutic methods that intend to increase the body immune

system's capacity to eliminate cancer cells without causing damaging results coming from overactivation or systemic poisoning [55]. Furthermore, the Western blot evaluation clarifies the intricacies of NK cell activation coupled with the possibility for targeted treatments to conquer the restrictions of standard activation approaches [56]. The differential healthy protein expression observed post-treatment highlights the elaborate network of signaling paths associated with NK cell features coupled with the opportunity of leveraging these paths to enhance healing results [57]. This method not just uses an extra innovative understanding of NK cell biology yet additionally blazes a trail for the growth of accuracy medication approaches in oncology [58].

In conclusion, based on this in vitro study, glucose culture is a very effective way to stimulate natural killer (NK) cells compared to the traditional cytokine-based stimulation in enhancing NK cell readiness and cytotoxicity against cancer cells. The significant increase of activation and cytotoxicity genes in NK cells after glucose culture as evidenced by the significant elevation of CD16, CD56, Granzyme B and perforin expression is indicative of how it works. Besides this method leads to an increase of CD69+ NK cells and an enhanced post-glucose activated cytolytic capability thereby making it more potential for improving the effectiveness of NK cell-based immunotherapy approaches used in cancer treatment.

Thus, from results obtained through flow cytometry and Western analysis techniques, our assertion that glucose culture can improve anticancer effects mediated by natural killer (NK) cells has been validated. It should be noted that Calcineurin A reduced following siRNA and Calcineurin inhibitors treatments justifies the fact that there exist strategies which could be applied to boost NK cell mediated cytotoxicity. This research further highlights the need for optimized methods for activating these cells towards maximal therapeutic benefits given their prominent role in innate immunity against cancers.

Current investigation proposes glucose culture as a viable alternative to address issues like immediate induction of immune responses against foreign antibodies in case current therapies are introduced including limited efficacy of cytokine activation among others pertaining to such therapies. Activating NK cells using this method not only exhibits higher efficiency but also hints at a consistent way that can be scaled up for boosting antitumor activities.

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## Conflict of interest

The author declares that there is no conflict of interest.

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**E-mail: hayfaa_fahad@aliraqia.edu.iq**
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