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Comparison of NK Cell Count Between 1% and 10% FBS Culture Media Concentration by FACS Analysis

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ABSTRACT

Recently, there has been a growing research interest in the application of immunotherapy for cancer treatment. Various approaches have been developed, and one of these methods involves utilizing Natural Killer (NK) cells of the immune system to specifically target cancer cells. Research on this method necessitates the mass and stable production of NK cells through culturing. For NK cell culture, Fetal Bovine Serum (FBS) is widely used in the industry at concentrations ranging from 10% to 20%. In an effort to explore a more efficient culturing technique, this study aims to investigate the effect of 1% FBS on cell viability of NK cells in cell culture. Because NK cells are usually defined by cell surface molecules, the efficacy of using a lower FBS concentration was quantitatively measured using Fluorescence-Activated Cell Sorting (FACS). The cell counts of cells cultured in 1% FBS was compared to those of cells cultured in 10% FBS. The results showed that no significant difference was found between cell count of cells cultured in 1% and 10% FBS. Thus, 1% FBS concentration culturing method was not found to be inferior. Further research is needed on the effect of replicating cancer microenvironment on NK cell viability.

Introduction

In contemporary oncology, immunotherapy is emerging as a promising and innovative tool in the medical field to treat cancer patients. Immunotherapy is categorized into two major groups: immune check point modulations and immune cell engineering therapies. Among immune cells, lymphocytes called NK cells have garnered interest due to their dual function in combating malignant cancer and modulating immune responses. Therapies involving NK cells directly adjust tumor microenvironment (TME), thereby leading to diverse prognoses. TME serves as a fundamental and theoretical arena in evaluating the effectiveness of therapy and surrounding cellular interactions. As a method of changing TME to enhance cancer cell response, the importance of NK cell differentiation and culturing from a patient's stem cell is gradually emerging. Over the past two decades, NK cell-mediated immunotherapy has become a safe and effective treatment for patients with advanced leukemia. Since then, the field of NK cell-based cancer therapy has grown exponentially, pioneering a major area of innovation in immunotherapy (Myers et al. 2020). Up to the cellular expansion step, NK cells are nurtured and buffered using various mixtures based on commercial FBS (Minetto et al. 2019). Thus, FBS clearly occupies a crucial position in determining the culture environment.

FACS

A flow cytometer is a testing apparatus capable of analyzing various characteristics such as the expression analysis of cell surface proteins, cell cycle, cell survival status, and cell size and shape. To analyze cells, artificial antibodies attached to fluorescent molecules must react with antigens on the cell surface (Wong et al. 2021). Measuring the fluorescence quantifies the antigens expressed on the cell surface.

NK Cells

NK cells or large granular lymphocytes (LGLs), are a type of cytotoxic lymphocyte that is important for the innate immune system. They belong to a rapidly expanding family of known innate lymphocyte cells (ILCs) and account for 5–20% of all circulating lymphocytes in humans (Arachchige et al. 2021). The role of NK cells is similar to that of cytotoxic T cells. NK cells provide a rapid response to virus-infected cells and other intracellular pathogens, acting approximately 3 days after infection, and they respond to tumorigenesis (Björkström et al. 2021).

FBS

Fetal bovine serum (FBS) is derived from blood collected from a bovine fetus through a closed collection system in a slaughterhouse. FBS is the most widely used serum supplement for the in vitro cell culture of eukaryotic cells. FBS concentration varies within each type of culture, usually ranging from 10% to 20% (Martin-Iglesias et al. 2021). In the NK cell industry, the ability to generate more NK cells from the same amount of peripheral blood is critical. So, the investigation of NK cell culture conditioning is a major interest in the field (De Jonge et al. 2023).

Overview: In this experiment, a FBS concentration of 1% was used instead of the conventional 10–20% to investigate its efficiency. During the preparation stage, anti-CD16 antibody coating is used as a stimulant for increased NK cell production (De Jonge et al. 2023) (Huang et al. 2020). The FBS concentration was set at 1% after peripheral blood mononuclear cell (PBMC) separation from blood during the initial stages of NK cell culture. The purpose of this study was to confirm the difference between using the frequently employed 10% FBS in general cell culture and the alternative use of 1% FBS. While NK cell culture usually takes about 14 days, the period is limited to 7 days for small-scale progression. The ratio of CD3+ and CD56+ cells was measured to confirm the progress of differentiation from PBMC to NK or T cells, and the survival rate of cells was determined through cell counters (Bi et al. 2020).

Methods

A 6-well plate was coated with anti-CD16 antibody and incubated in an incubator at 37°C for 2 hours. This treatment acts as a stimulating factor for NK cell proliferation and activation. PBMCs at a concentration of 5 x 10^6 cells per well were seeded into a 6-well plate (Fig.1). NK cells were cultured under conditions of 1% FBS and 10% FBS (Fig.1). After 5 days, the cells were transferred to a T25 flask, and the medium changed on day 3. On day 5, 5mL of media was added to reach a total volume of 10mL. Then the total cells harvested were analyzed on day 7 (Fig.2). After culturing for day 3, 5, and 7 under each media condition, cells were harvested and analyzed for viability and CD3/CD56 expression ratio using FACS (Table.1). The proportion of NK cells was determined using a flow cytometer with anti-CD3 antibody (FITC) and CD56 antibody (APC) under conditions shown in table 2. Antibody staining was carried out for 30 minutes at 4°C and washing was performed three times with antibodies of distinct color and concentration (Table.3). The cell number was adjusted to a concentration of 2 x 10^5 cells in phosphate buffered saline (PBS) and then stained and analyzed.

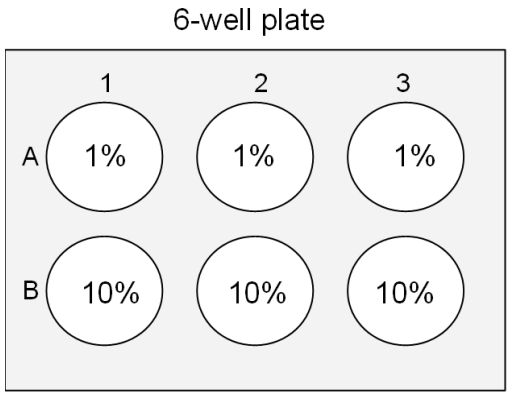

Figure 1. Columns 1, 2, and 3 represent cells cultured for day 3, 5 and 7, respectively. Row A consists of cells cultured in 1% FBS, while Row B consists of cells cultured in 10% FBS.

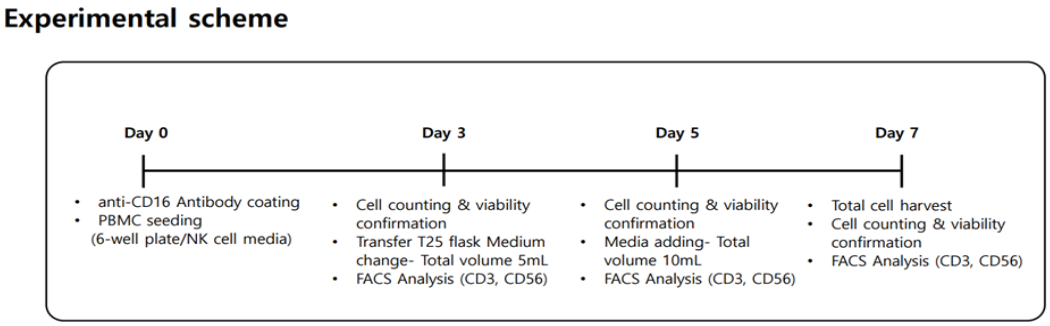

Figure 2. Visual outline of the experiment

Table 1. Experiment schedule and procedure

Day	Experiment procedure
Day 0	CD16 AB coating 2 hours in 37°C incubator PBMC 5 x 10^6 cells/5mL/well seeding (6-well plate) NK cell media + 1% or 10% FBS condition
Day 3	Harvest -> cell viability confirmation & CD3, CD56 expression confirmation (FACS) Media change 5mL in T25 flask
Day 5	Some harvest -> cell viability confirmation & CD3, CD56 expression confirmation (FACS) Add media 5mL (Total volume 10mL)
Day 7	Total cell harvest -> cell viability confirmation & CD3, CD56 expression confirmation (FACS)

Table 2. Staining conditions

Conditions	
Date	Day 3, 5, 7
Staining	2 x 10^5 cells/DPBS (100uL) Staining 4°C 30 minutes Washing 3 times

Table 3. Antibody colors and details

Category	CD3	CD56	CD3 Iso-type	CD56 Isotype
Color	FITC	APC	FITC	APC
Isotype	IgG1	IgG2b	IgG1	IgG2b
Ab Cat. No.	555332	341025	555748	555745
2x10^5 cells/ DPBS 100uL	4 uL	1 uL	4 uL	4 uL

Results

After treating each culture unit, cellular fields showing cell numbers and viability were initially inspected under a microscope with 4x and10x magnification on day 2, 3, 5, and 7. The cell survival rate and NK cell proportions were confirmed through FACS anti-CD3/CD56 analysis.

Cell Number & Viability

Microscope Photos (4x and 10x)

Microscopic evaluation clearly shows the formation of well-defined cell clusters on each day (Fig.3, Fig.4). When observed under a microscope, cluster formation and size were shown to be better in 10% FBS than in 1% FBS.

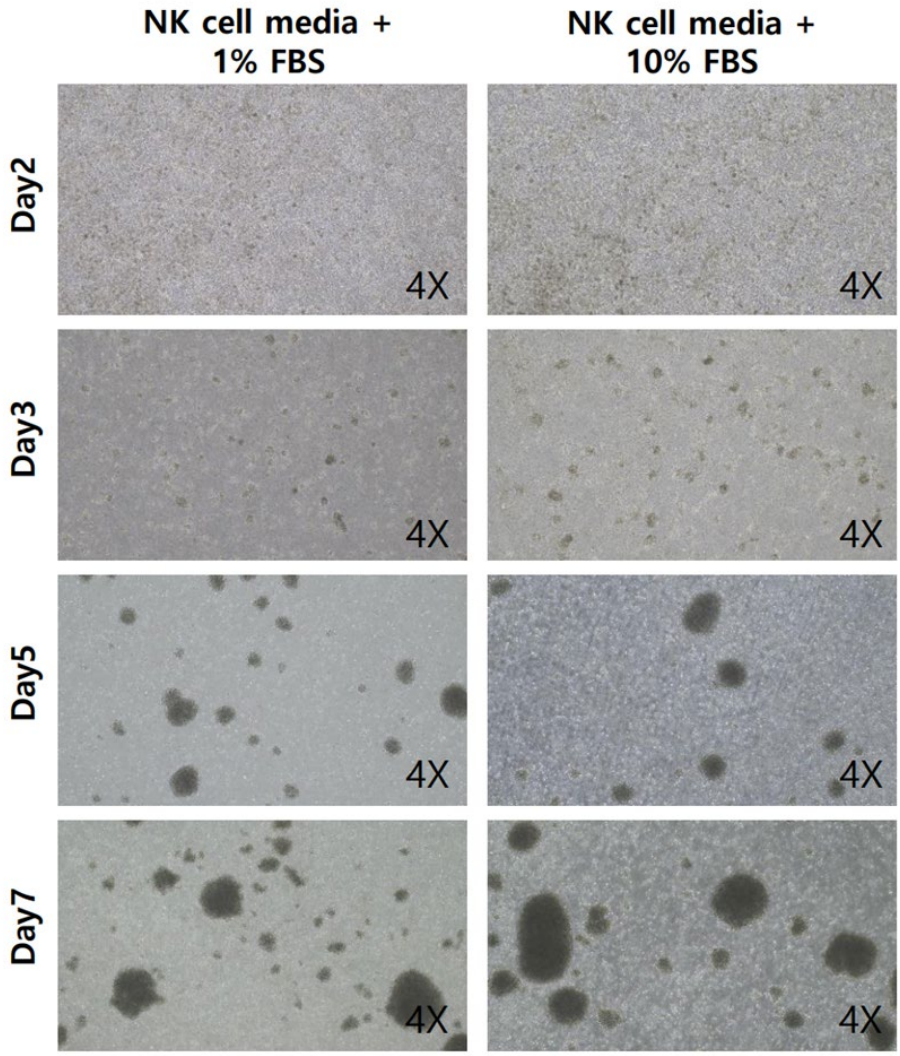

Figure 3. Cell images taken on day 2, 3, 5, and 7 using a 4x magnification lens microscope

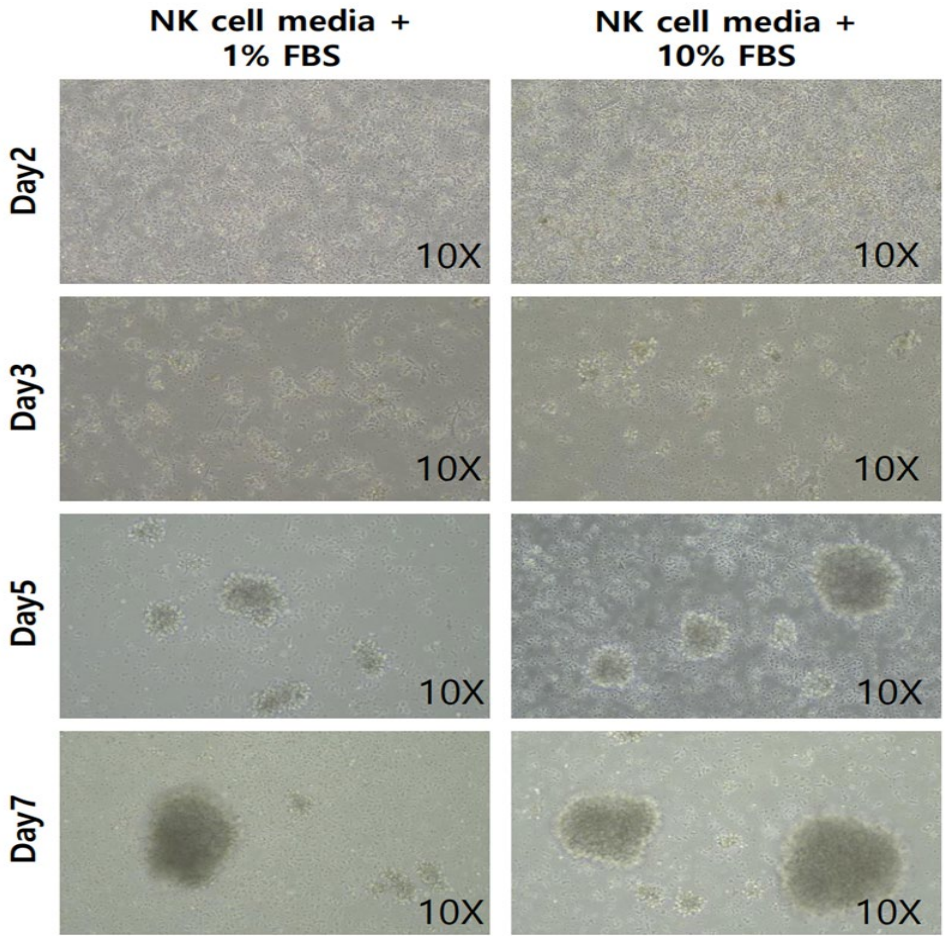

Figure 4. Cell images taken on day 2, 3, 5, and 7 using a 10x magnification lens microscope

Cell count and survival rate results were measured using cell counter instruments. The total cell count is the sum of dead and live cells, measured in cells/mL. Cell counts and survival rates did not differ significantly (Table.4). The difference was only within 10%. The proportion of NK cells increased progressively by day. The proportion of NK cells did not differ significantly between 1% and 10% culture conditions (Table.5).

Table 4. Live cell counts at each time point

Day	NK cell media	Total (cells/mL)	Live cell (cells/mL)	Viability (%)	Volume (mL)
Day 3	1% FBS	7.13×10^5	5.83×10^5	81.28	5mL
	10% FBS	6.18×10^5	5.01×10^5	81.27	
Day 5	1% FBS	1.95×10^6	1.59×10^6	82.53	

	10% FBS	1.40 x 10^6	1.14 x 10^6	81.77	
Day 7	1% FBS	4.16 x 10^6	3.01 x 10^6	72.14	
	10% FBS	3.58 x 10^6	2.82 x 10^6	78.92	

Table 5. NK cell proportion by timeline (highlighted in red)

Day	NK cell media	CD3+ CD56- (%)	CD3- CD56+ (%)	CD3+ CD56+ (%)
Day 3	1% FBS	19.05	58.76	4.02
	10% FBS	16.95	51.89	3.93
Day 5	1% FBS	15.77	76.33	3.83
	10% FBS	16.84	75.09	3.93
Day 7	1% FBS	9.27	84.86	4.53
	10% FBS	11.91	81.83	4.55

NK Cell Confirmation (FACS)

Day 3 Flow Cytometer Measurement Results: The graph on the right, labelled CD3 CD56, utilizes the horizontal axis for cells expressing CD56 and the vertical axis for cells expressing CD3. The isotype is used as the control group to assess the relative expression levels of CD3 and CD56. The boxes highlighted in light blue show cells that are CD3- and CD56+, which are NK cells.

The CD3-, CD56+ ratio is approximately 5–10% higher under the 1% FBS condition (Fig.5). No significant difference was observed in cell viability (Fig.5).

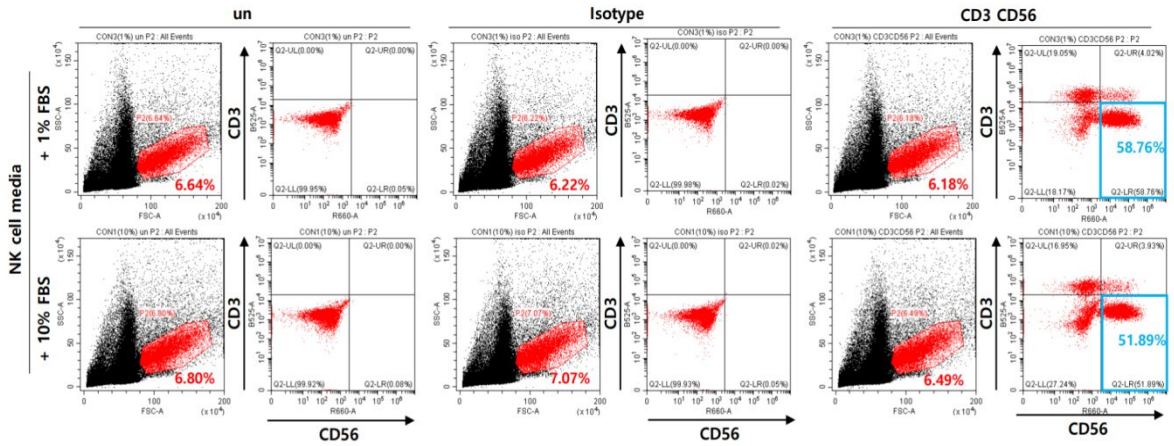

Figure 5. FACS results for day 3. The NK cell portion is represented by the blue squares. The three graphs depict unstained cells, isotype control cells, and cells stained with anti-CD3 and CD56 antibodies. The top row shows the results for cells grown in 1% FBS media, while the bottom row shows the results for cells grown in 10% media.

Day 5 Flow Cytometer Measurement Results: The method of organizing the results is the same as on the previous section displaying day 3 FACS results. There is no significant difference in CD3-, CD56+ ratios across all experimental groups (Fig.6).

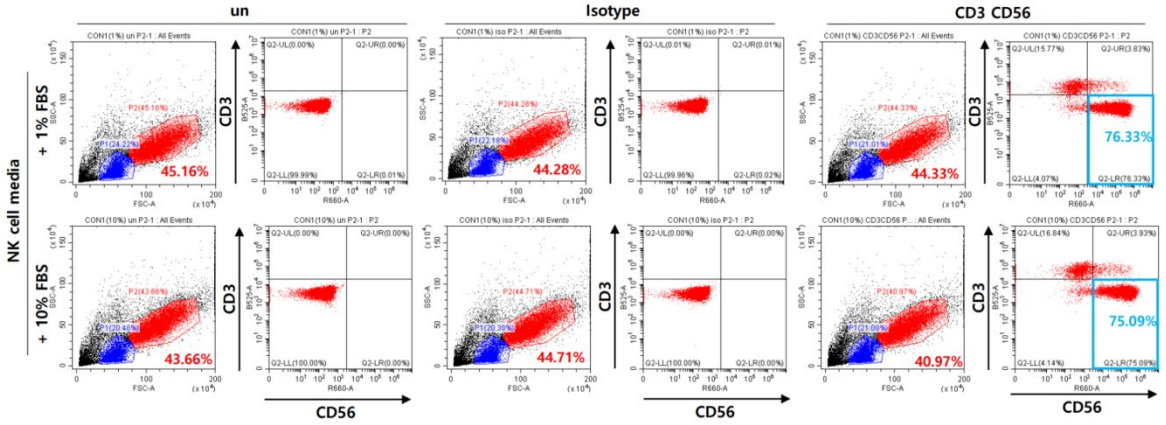

Figure 6. FACS results for day 5. The NK cell portion is represented by the blue squares. The three graphs depict unstained cells, isotype control cells, and cells stained with anti-CD3 and CD56 antibodies. The top row shows the results for cells grown in 1% FBS media, while the bottom row shows the results for cells grown in 10% media.

Day 7 Flow Cytometer Measurement Results: The method of organizing the results is the same as on the previous section displaying day 5 FACS results. There is no significant difference in CD3-, CD56+ ratios across all experimental groups (Fig.7).

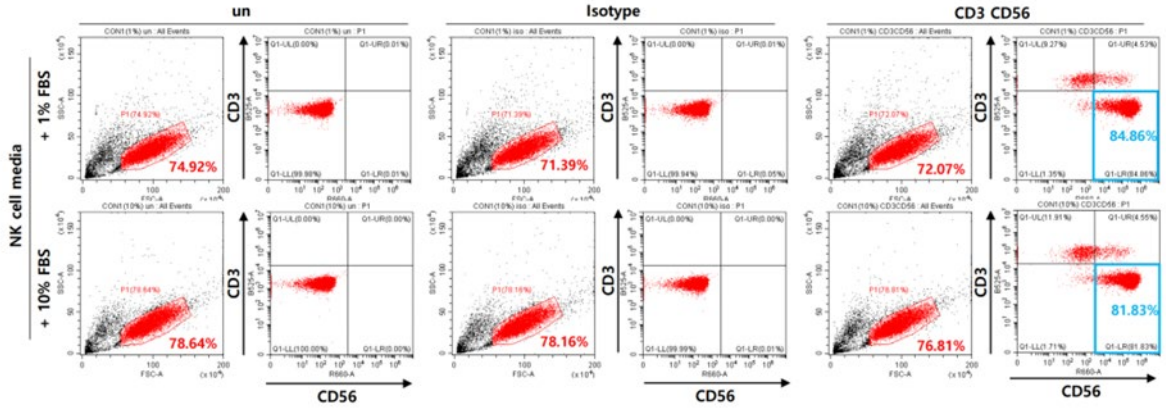

Figure 7. FACS results for day 7. The NK cell portion is represented by the blue squares. The three graphs depict unstained cells, isotype control cells, and cells stained with anti-CD3 and CD56 antibodies. The top row

shows the results for cells grown in 1% FBS media, while the bottom row shows the results for cells grown in 10% media.

Discussion

In this experiment comparing 1% vs. 10% FBS media cultures on day 3, 5, and 7, no significant difference was observed in total cell numbers, viability, or NK cell ratio. The overall production of NK cell counts did not show a significant difference between the 1% and 10% FBS environments. If there is adequate culture media refreshment, a 1% FBS concentration could be sufficient for NK cell production. Using less FBS would be more economical without introducing potential bias to the study.

NK cells can be readily identified in cancer tissues, where interactions occur among cancer cells, fibroblasts, adipose cells, and various leukocytes (Cózar et al. 2021). Clearly, NK cells function as leukocyte recruiters, tumor suppressors, immune regulators, and direct cancer cell killers (Yao et al. 2023) (Crinier et al. 2020) (Jiang et al. 2023). There are more advanced NK cell activity and affinity enhancers such as CAR-NK technology (Gong et al. 2021) (Islam et al. 2021) (Mantesso et al. 2020). Molecular interactions within cell function-related cascades sometimes exhibit reciprocal curves (Frutoso et al. 2019). NK cell production is controlled by maturation factors and stimulators (Kweon et al. 2019) (Portale et al. 2023). Sometimes, NK cells function in different environments such as lymph nodes or blood circulation (Rethacker et al. 2022) (Vyas et al. 2023).

In each cancer microenvironment, NK cells can assume different roles or show varying levels of activity (Hodgins et al. 2019) (Nersesian et al. 2023) (Wu et al. 2020). As such, it is not easy to distinguish which microenvironment or cancer gene expression profile is the target for NK therapy (Minetto et al. 2019) (Myers et al. 2020). The limitations of NK therapy can be overcome by in vivo experiments and molecular studies of cancer microenvironments (Parodi et al. 2023) (Vogler et al. 2021). Cultivating NK cells in a culture environment that can potentially mimic the various types of TME would be costly. Lowering FBS concentration is expected to reduce the associated costs.

Conclusion

The NK cell production under the 1% FBS culture media condition did not yield NK cell production that is significantly different from the conventional concentration of 10%. The overall live NK cell count and types were not significantly different from those cultured in 10% FBS media.

Limitations

This experiment performed a preliminary analysis of the efficacy of 1% FBS media for NK cell production. The experiment was conducted using a single bottle (with each analysis repeated 3 times) and multiple trials were not performed per group. Conducting multiple trials would further substantiate the significance of the results and the efficacy of 1% FBS.

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References

Arachchige, A. S. P. M. (2021). Human NK cells: From development to effector functions. Innate Immunity, 27(3), 212–229. https://doi.org/10.1177/17534259211001512

Bi, J., & Wang, X. (2020). Molecular regulation of NK cell maturation. Frontiers in Immunology, 11. https://doi.org/10.3389/fimmu.2020.01945

Björkström, N. K., Strunz, B., & Ljunggren, H. G. (2021). Natural killer cells in antiviral immunity. Nature Reviews Immunology, 22(2), 112–123. https://doi.org/10.1038/s41577-021-00558-3

Cózar, B., Greppi, M., Carpentier, S., Narni-Mancinelli, É., Chiossone, L., & Vivier, É. (2021). Tumor-Infiltrating natural killer cells. Cancer Discovery, 11(1), 34–44. https://doi.org/10.1158/2159-8290.cd-20-0655

Crinier, A., Narni-Mancinelli, É., Ugolini, S., & Vivier, É. (2020). SnapShot: Natural Killer Cells. Cell, 180(6), 1280-1280.e1. https://doi.org/10.1016/j.cell.2020.02.029

De Jonge, P., Van Hauten, P. M., Janssen, L. M. A., De Goede, A. L., Berrien-Elliott, M. M., Van Der Meer, J. M., Mousset, C. M., Roeven, M. W., Foster, M., Blijlevens, N. M. A., Hobo, W., Fehniger, T. A., Jansen, J. H., Schaap, N., & Dolstra, H. (2023). Good manufacturing practice production of CD34+ progenitor-derived NK cells for adoptive immunotherapy in acute myeloid leukemia. Cancer Immunology, Immunotherapy, 72(10), 3323–3335. https://doi.org/10.1007/s00262-023-03492-6

Frutoso, M., & Mortier, E. (2019). NK cell hyporesponsiveness: More is not always better. International Journal of Molecular Sciences, 20(18), 4514. https://doi.org/10.3390/ijms20184514

Gong, Y., Wolterink, R. G. J. K., Wang, J., Bos, G. M., & Germeraad, W. T. (2021). Chimeric antigen receptor natural killer (CAR-NK) cell design and engineering for cancer therapy. Journal of Hematology & Oncology, 14(1). https://doi.org/10.1186/s13045-021-01083-5

Hodgins, J. J., Khan, S., Park, M. M., Auer, R. C., & Ardolino, M. (2019). Killers 2.0: NK cell therapies at the forefront of cancer control. Journal of Clinical Investigation, 129(9), 3499–3510. https://doi.org/10.1172/jci129338

Huang, Z., Blum, R., Bjordahl, R., Gaidarova, S., Rogers, P., Lee, T. T., Abujarour, R., Bonello, G., Wu, J., Tsai, P., Miller, J. S., Walcheck, B., Valamehr, B., & Kaufman, D. S. (2020). Pluripotent stem cell–derived NK cells with high-affinity noncleavable CD16a mediate improved antitumor activity. Blood, 135(6), 399–410. https://doi.org/10.1182/blood.2019000621

Islam, R., Pupovac, A., Evtimov, V., Boyd, N., Shu, R., Boyd, R., & Trounson, A. (2021). Enhancing a natural killer: Modification of NK cells for cancer immunotherapy. Cells, 10(5), 1058. https://doi.org/10.3390/cells10051058

Jiang, H., & Jiang, J. (2023). Balancing act: the complex role of NK cells in immune regulation. Frontiers in Immunology, 14. https://doi.org/10.3389/fimmu.2023.1275028

Kweon, S., Phan, M. T., Chun, S., Yu, H., Kim, J., Kim, S., Lee, J., Ali, A. K., Lee, S. H., Kim, S., Doh, J., & Cho, D. (2019). Expansion of human NK cells using K562 cells expressing OX40 ligand and short exposure to IL-21. Frontiers in Immunology, 10. https://doi.org/10.3389/fimmu.2019.00879

Mantesso, S., Geerts, D., Spanholtz, J., & Kučerová, L. (2020). Genetic engineering of natural killer cells for enhanced antitumor function. Frontiers in Immunology, 11. https://doi.org/10.3389/fimmu.2020.607131

Martin-Iglesias, S., Herrera, L., Santos, S., Vesga, M. Á., Eguizábal, C., Lanceros-Méndez, S., & Silván, U. (2023). Analysis of the impact of handling and culture on the expansion and functionality of NK cells. Frontiers in Immunology, 14. https://doi.org/10.3389/fimmu.2023.1225549

Minetto, P., Guolo, F., Pesce, S., Greppi, M., Obino, V., Ferretti, E., Sivori, S., Genova, C., Lemoli, R. M., & Marcenaro, E. (2019). Harnessing NK cells for cancer treatment. Frontiers in Immunology, 10. https://doi.org/10.3389/fimmu.2019.02836

Myers, J. A., & Miller, J. S. (2020). Exploring the NK cell platform for cancer immunotherapy. Nature Reviews Clinical Oncology, 18(2), 85–100. https://doi.org/10.1038/s41571-020-0426-7

Nersesian, S., Carter, E. B., Lee, S. N., Westhaver, L. P., & Boudreau, J. E. (2023). Killer instincts: natural killer cells as multifactorial cancer immunotherapy. Frontiers in Immunology, 14. https://doi.org/10.3389/fimmu.2023.1269614
Parodi, M., Astigiano, S., Carrega, P., Pietra, G., Vitale, C., Damele, L., Grottoli, M., De La Luz Guevara Lopez, M., Ferracini, R., Bertolini, G., Roato, I., Vitale, M., & Orecchia, P. (2023). Murine models to study human NK cells in human solid tumors. Frontiers in Immunology, 14. https://doi.org/10.3389/fimmu.2023.1209237

Portale, F., & Di Mitri, D. (2023). NK cells in cancer: mechanisms of dysfunction and therapeutic potential. International Journal of Molecular Sciences, 24(11), 9521. https://doi.org/10.3390/ijms24119521

Rethacker, L., Boy, M., Bisio, V., Roussin, F., Denizeau, J., Vincent-Salomon, A., Borcoman, É., Sedlik, C., Piaggio, E., Toubert, A., Dulphy, N., & Caignard, A. (2022). Innate lymphoid cells: NK and cytotoxic ILC3 subsets infiltrate metastatic breast cancer lymph nodes. OncoImmunology, 11(1). https://doi.org/10.1080/2162402x.2022.2057396

Vogler, M., Shanmugalingam, S., Särchen, V., Reindl, L. M., Grèze, V., Buchinger, L., Kühn, M. W., & Ullrich, E. (2021). Unleashing the power of NK cells in anticancer immunotherapy. Journal of Molecular Medicine, 100(3), 337–349. https://doi.org/10.1007/s00109-021-02120-z

Vyas, M., Requesens, M., Nguyen, T. H., Peigney, D., Azin, M., & Demehri, S. (2023). Natural killer cells suppress cancer metastasis by eliminating circulating cancer cells. Frontiers in Immunology, 13. https://doi.org/10.3389/fimmu.2022.1098445

Wu, S., Fu, T., & Jiang, Y. (2020). Natural killer cells in cancer biology and therapy. Molecular Cancer, 19(1). https://doi.org/10.1186/s12943-020-01238-x

Yao, L., Hou, J., Xin, W., Lu, Y., Jin, Z., Zhou, Y., Yu, B., Li, J., Yang, Z., Li, C., Yan, M., Zhu, Z., Liu, B., Yan, C., & Su, L. (2023). Cancer-associated fibroblasts impair the cytotoxic function of NK cells in gastric

cancer by inducing ferroptosis via iron regulation. Redox Biology, 67, 102923. https://doi.org/10.1016/j.redox.2023.102923

Wong, P., Wagner, J. A., Berrien-Elliott, M. M., Schappe, T., & Fehniger, T. A. (2021). Flow cytometry-based ex vivo murine NK cell cytotoxicity assay. *STAR Protocols*, *2*(1), 100262. https://doi.org/10.1016/j.xpro.2020.100262