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Study on the Standardized Preparation of NK Cells and the Mechanism of Anti-aging of Rhesus Monkey PBMC

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ABSTRACT

Background: Natural killer (NK) cells are an important part of the immune system and play a central role in cell-mediated immune responses. Recent studies have shown that NK cells promote organ rejuvenation, thus achieving anti-aging effects from the inside to out.

Methods: We amplified and cultured human peripheral blood NK cells and the positive rate reached 43.91%. After purification, the positive rate reached 92.65%. The cultured NK showed a good killing effect on tumor cells and the NK cells reached the standard preparation scale. The NK cells cultured by us were co-cultured with the PBMC of aging macaques and the quantitative PCR and WB results showed that the expression of aging gene and aging protein of the PBMC of aging macaques decreased significantly after co-culture, with statistical significance ($P<0.01$).

Result: These results indicate that NK cells prepared with standard preparation have anti-aging effect, which will have extensive significance in preclinical research.

Key words: Aging gene, Anti-aging, Cell killing experiment, NK cells, Senescence protein, Standardized preparation.

INTRODUCTION

NK cells belong to innate immune cells with an immunophenotype characteristic of CD3-/CD56+, which is a lymphocyte subgroup that does not express T cell receptors or B cell receptors (Wright and Bonavida, 1982). NK cells can kill tumor cells, virus-infected cells and injured cells without being restricted by MHC and have the ability to secrete a variety of cytokines (Lotzova *et al*., 1987; Warren, 1984). Therefore, NK cells are considered to be the first line of defense against infection and tumor in the body, with functions of anti-tumor, anti-infection and immune regulation (Sarun *et al*., 1998; Scott-Algara *et al*., 1992). In particular, NK cells play an important role in tumor immunity and anti-aging, adoptive NK cell immunotherapy becomes a new method for tumor treatment and anti-aging (Baraz *et al*., 1999; Lindberg *et al*., 1999).

However, the main problem facing NK cell immunotherapy technology is how to improve the efficiency and purity of cell expansion. Therefore, it is necessary to optimize and improve the preparation system of NK cells in order to improve the purity and activity of NK cells. The anti-aging effect of NK cells needs further study. Quantitative PCR and WB detection of age-related genes and proteins can intuitively reflect the anti-aging effect of NK cells and more clearly study the anti-aging mechanism of NK cells.

Studies have linked aging cells to chronic inflammation and age-related diseases such as atherosclerosis, osteoarthritis and even diabetes. Senescent cells accumulate throughout the body as we age, but senescent immune cells are most at risk and are the primary cells that accelerate aging throughout the body. Therefore, targeted elimination or modification of such cells is the key to the success of reversing aging and extending life. The nature of NK cells determines that it has anti-aging effects.

In this study, Human NK cells were cultured and purified with stem cell's EasySep Human NK Cell Iso Kit. The ability of NK cells to kill tumor cells was tested and the obtained NK was co-cultured with PBMC of aging macaques. Quantitative PCR and WB results showed that the expression of aging genes and aging proteins in PBMC of aging macaques decreased after co-culture, indicating that the anti-aging mechanism of NK cells starts from reducing the expression of aging genes and proteins.

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MATERIALS AND METHODS

Isolation, amplification and culture of NK cells

We performed the NK cell culture with the Tonglihaiyuan derived NK cell culture amplification kit. According to the operation instructions, 1000 mL cell suspension was harvested on the 14th and 15th day and the total number of cells reached 4-9×10^9. The experimental study was endorsed by the 920th Hospital Ethics Committee of the Joint Logistic Support Force. The obtained cells were labeled with flow cytometry antibody and the percentage of CD3-CD56+ was observed to be 43.91%. The experiment will be conducted in the Basic Medical Laboratory of the 920th Hospital of the Joint Logistics Support Force of PLA from 2022 to 2023.

NK cell purification

Purified with stem cell's EasySep Human NK Cell Iso Kit. NK cells were purified according to the instructions of the kit and the purity of NK cells reached more than 90% after purification.

NK cells kill tumor cells

NK cells adjusted the cell concentration to 2×10^6/ml, 50 ul per well, *i.e.* 1×10^5 cells per well, adding 1×10^4 target cells, the effect target ratio was 10:1. The NK cells were diluted 1 times, 1×10^6 / ml, 50 μl per well, *i.e.* 5×10^4 cells per well and 1×10^4 target cells were added, the effect target ratio was 5:1. Then dilute 5 times, 2×10^5/ml, 50 ul per well, that is, 1×10^4 cells per well, add 1×10^4 target cells, effect target ratio 1:1, add 3 multiple Wells. NK cell natural release holes, add 1×10^4, 5×10^4, 10×10^4, 50 μl per well, 3 multiple holes.

K562 were adjusted the cell concentration to 2×10^5/ml and 50 ul per well, so the number of cells per well was 1×10^4. The number of natural release pores in K562 cells was 1×10^4 and 3 multiple pores per well. The number of natural release pores in medium was 100 ul and 3 multiple pores per well. The maximum number of release pores in K562 cells was 1×10^4 and 3 multiple pores per well.

The cells were incubated at 37°C with 5% CO_2 for 4 h. 10 μl lysate was added into each maximum release well 45 min before the end of the reaction. After the reaction, 50 μl supernatant and 50 μl LDH enzyme reaction solution were absorbed from each well and placed in a new 96-well plate for 30 min at room temperature and 50 μl reaction stop solution was added. The D value was detected by enzyme marker. Calculation formula:

$$\text{Killing activity (\%)} = \frac{\text{Determination tube D value - Target cell natural release tube D value - Effector cell natural release tube D value}}{\text{Maximum target cell release tube D value - Target cell natural release tube D value}} \times 100\%$$

We used the newly isolated PBMC, cultured NK cells and purified NK cells to kill K562 cells respectively. The results showed that the purified NK cells had the strongest killing activity.

Co-culture of NK cells and PBMC of aging macaques

10 ml blood was collected from aged macaques and PBMC was isolated. 2.6 ml NK cells were added below the co-culture chamber and only medium was added for the control. 1.5 ml PBMC of aging macaque was added to the top and the cell concentration was 2×10^6 / ml. After 3 days of co-culture, upper PBMC was absorbed for quantitative PCR detection of age-related genes and WB detection of age-related proteins. The primer sequence of the gene is shown in Table 1. WB antibody was purchased from CST.

RESULTS AND DISCUSSION

Flow cytometry detection of cultured NK cells (Fig 1)

The purity of NK cells reached 43.91% after culture.

NK cells killing K562 cells experiment (Fig 2)

We used the newly isolated PBMC, cultured NK cells and purified NK cells to kill K562 cells respectively. The results showed that when the effect-to-target ratio was 1:1, 5:1 and 10:1, the percentage of killing K562 cells of cultured NK cells and purified NK cells was significantly higher than that of PBMC. The results were statistically significant

Table 1: Primers sequence of aging related genes in rhesus monkeys.

Primer information	Primer name	Primer sequence (5′-3′)	Segment length (bp)	Annealing temperature (°C)
NM_001047151.2	macaque-P53-S	CCTCAGCACCTTATCCGAGTG	256	60.2
	macaque-P53-A	CACAAACTCGCACCTCAAAGC		60.6
XM_015137250.2	macaque-SOD2-S	ACAAATTGCTGCTTGTCCGAA	102	59.32
	macaque-SOD2-A	TACTGAAGGTAATAAGCGTGCTCC		60.44
XM_015139582.2	macaque-CASP3-S	GGAACAAATGGACCTCTTGACCT	137	60.5
	macaque-CASP3-A	GTCTCAATGCCACAGTCCAGTTC		61.66
NM_001261016.2	macaque-BAX-S	GACGGCAACTTCAACTGGG	92	59.05
	macaque-BAX-A	AGTTCGGGCACCTTGGTACAC		61.3
XM_028838050.1	macaque-BCL2-S	CCCTGTGGATGACTGAGTACCTG	91	62
	macaque-BCL2-A	GGGCCGTACAGTTCCACAAA		60.54

(*P<0.05, # P<0.01), indicating that the cultured NK cells had significant killing activity against tumor cells (Fig 2). The successful preparation of NK cells provided reliable seed cells for anti-tumor research.

NK cells formed colonies after amplification and GFP transfected with green fluorescence (Fig 3)

After successful transfection of GFP, it can be used for *in vivo* tracer of NK cells when transplanted *in vivo*.

Co-culture of NK cells with aging rhesus monkey PBMC

After co-culture of human NK cells with monkey PBMC, quantitative PCR detected decreased expression of age-related genes (Fig 4).

After co-culture of human NK cells with monkey PBMC, WB detected decreased expression of age-related proteins (Fig 5).

China's elderly population is growing rapidly and it is estimated that by 2050, one third of the country's population will be over 60 years old (Foreman *et al*., 2018). To some extent, human aging is a natural law. As time goes by, the functions of various organs gradually decline, which is exactly a kind of disease from the perspective of biomedicine (Luukkainen *et al*., 2018; Ma *et al*., 2020). If we can delay the decline of human functions, then we have achieved the purpose of curing aging. Of course, we do not violate the laws of nature, but can reach the aging degree of delay.

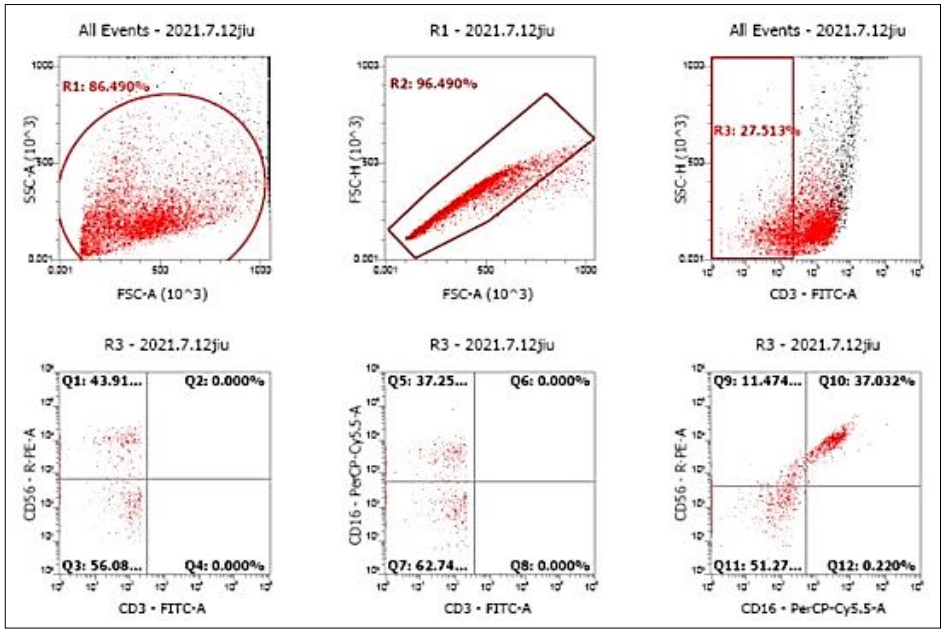


Fig 1: Purity of NK cells reached 43.91% after culture.

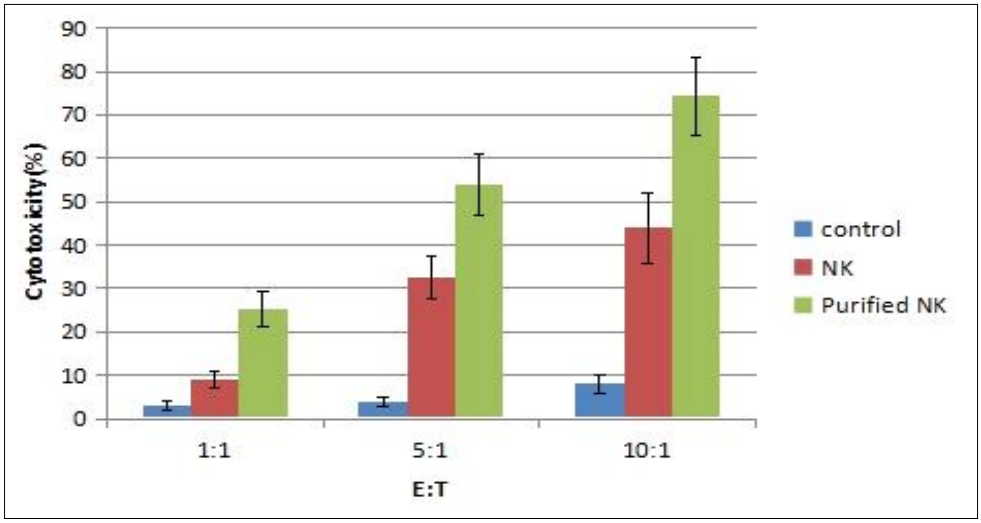
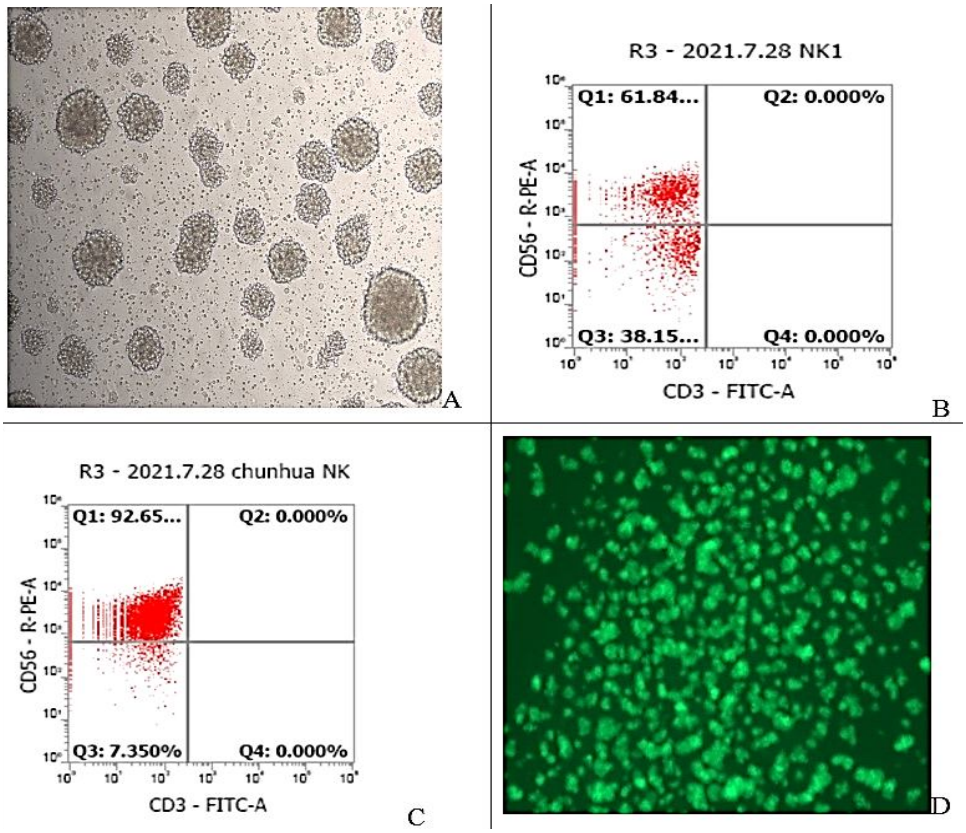
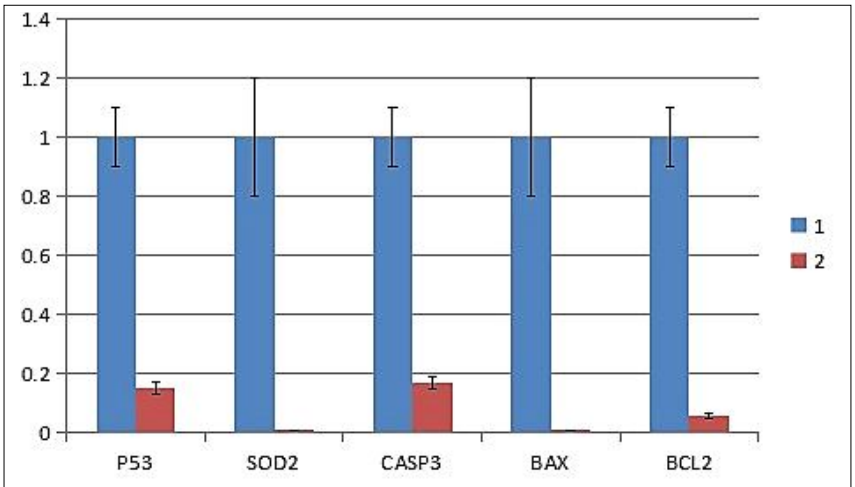


Fig 2: PBMC, comparison of killing activity of cultured NK cells and purified NK cells (*P<0.05, #P<0.01, compared with PBMC group).



A: Growth morphology of NK cells (100×). We used the same sea source NK cell culture box. After 5 days of culture, the cells formed a large number of colonies and the cell proliferation was good. B: Flow cytometry of NK cells. Flow cytometry showed that the positive rate of NK cells was 61.84% and the positive rate of CD56+CD3- was 61.84%, which was consistent with the phenotypic characteristics of NK cells. C: Flow analysis of NK cells after purification. We purified cultured NK cells with NK purification kit. The positive rate of NK cells was 61.84% before purification, 92.65% after purification and 92.65% for cell surface marker CD56+CD3-. D: GFP-labeled NK cells (100×). After GFP labeling, most NK cells showed bright green fluorescence under microscope excitation.

Fig 3: Growth morphology, immunophenotype and GFP markers of NK cells.



After co-culture, the expression of age-related genes P53, SOD2, CASP3, BAX and BCL2 decreased, indicating the anti-aging effect of human NK (the result was mean ± collimation difference, n=3). 2 compared with 1, the difference was statistically significant, P<0.01).

Fig 4: 1 is the cultured monkey PBMC, 2 is the monkey PBMC co-cultured with human NK.

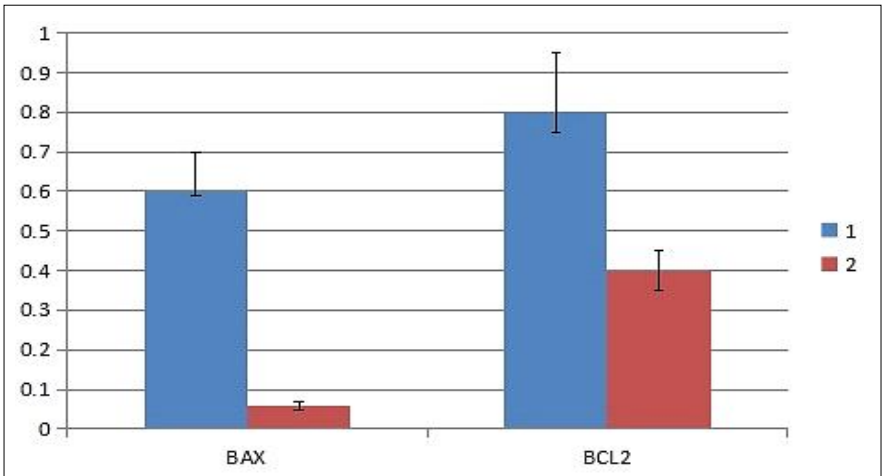


Fig 5: No. 1 is the PBMC of aging macaques and No. 2 is the PBMC of monkeys co-cultured with human NK, which is related to aging after co-culture.

Natural killer (NK) cells are an important part of the immune system and play a central role in cell-mediated immune responses. Understanding the changes of NK cells in the aging process is an important basis for delaying immune aging and strengthening the firewall of the elderly. At present, more and more studies have proved that immune cells play an important role in the aging process (Huang *et al*., 2018).

Recent studies have shown that NK cells can promote organ rejuvenation, thus achieving anti-aging effect from the inside to out (Basar *et al*., 2020; Bergman *et al*., 2020). In addition to eliminating transformed cells, NK cells are involved in many other biological processes, such as immune regulation, antimicrobial immune response and recognition and elimination of senescent cells (Daher and Rezvani, 2021; Morgan *et al*., 2020). However, obtaining sufficient quantity and purity of NK cells *in vitro* has always been a hot topic for scientists to study. The anti-tumor effect of NK cells has been confirmed, but the anti-aging effect and mechanism of NK cells need to be further studied.

CONCLUSION

The main challenge now is to identify markers of aging in NK cells, ultimately identify molecular targets that interfere with the aging process and translate these findings into anti-aging studies.

Through co-culture of NK cells with PBMC of aging macaques, we found that the expression of age-related genes and proteins decreased after co-culture, which confirmed the anti-aging effect of NK cells. This will have wide implications in preclinical research.

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Author contributions

Guang-Ping Ruan and Xiang Yao made substantial contributions to the study conception and design, data acquisition and data analysis and interpretation.

Guang-Ping Ruan, Feng Yan and Jing Gao conducted the experiments.

Xiang-Yu Feng and Tao Ye agree to be accountable for all aspects of the work and ensure that questions related to the accuracy or integrity of any part of the study will be appropriately investigated and resolved.

Xing-Hua Pan and Guang-Ping Ruan provided final approval of this version of the manuscript for publication.

Guang-Ping Ruan, Xing-Hua Pan, Feng Yan and Tao Ye were involved in drafting the manuscript or revising it critically for important intellectual content.

All authors read and approved the final manuscript.

Data availability

All data generated or analysed during this study are included in this published article.

Ethics declarations

All experiments were performed in accordance with relevant named guidelines and regulations. The authors complied with the ARRIVE guidelines. This study protocol was reviewed and approved by [the 920th Hospital of the Joint Logistics Support Force of PLA], approval number [2022-022-01]. Written informed consent was obtained from donors to participate in the study.

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Conflict of interest

The authors declare that they have no competing interests.

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