OPTIMIZATION OF THE FLOW CYTOMETRIC METHOD FOR ANALYZING NK CELL CYTOTOXIC ACTIVITY IN BREAST CANCER

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Abstract

Objectives: To establish a protocol for analyzing NK cell cytotoxic activity (NKAc) in breast cancer patients using the flow cytometric technique. ***Methods:*** We first optimized the protocol based on the previously published studies, focusing on choosing fluorescent dye concentration, incubation time length, and Effector:Target cell ratio (E:T ratio). Subsequently, we preliminarily applied the established protocol to characterize NKAc in some breast cancer patients and healthy controls. Lastly, we compared the NKAc versus NK cell secretory activity (NKAs) data obtained from the study. ***Results:*** The fluorescent dye concentration could be used according to the manufacturer's suggestion (CFSE: 2.5 - 10µM; Zombie NIR: 1:1000 - 1:100 dilution). The co-culture period of effector and target cells and the E:T ratio could be 4 hours and 5:1, respectively. NKAc was reproducible for 1 month. 2/7 breast cancer patients had NKAs < 200 pg/mL and NKAc < 10%. ***Conclusion:*** It is feasible to examine NKAc in breast cancer patients via the flow cytometric method established in this study. Further studies are needed to validate the diagnostic and prognostic value of NK cell function tests.

Keywords: NK cell cytotoxic activity; NK cell secretory activity; Flow cytometry; Breast cancer.

INTRODUCTION

NK cells, an important population of the innate immune system, protect the body against viruses and malignant cells. Infected cells or malignant cells often express activating ligands and reduce inhibitory ligands for the counterpart receptors of the NK cells, which stimulates

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NK cells to perform two major NK cell functions: NKAc and NKAs. It has been well documented that NKAc and NKAs were suppressed in a subgroup of cancer patients, especially in prostate, lung, colon, and breast cancer, though the mechanism of these observations has not been clarified yet [1, 2, 3, 4, 5]. The effectiveness of NK cell therapy in cancer could be indicated by clinical and immunological responses. Therefore, it is important to have a good system to quantify the activity of NK before and after NK cell therapy in cancer patients. In terms of NKAs, there is an IVD assay to measure the level of IFN-γ secretion of Promoca (engineered recombinant cytokines)-stimulated pNK via ELISA availably [6]. There is currently no standardized commercial assay to measure NKAc; therefore, each laboratory must set up its own protocol and document the reference range of NKAc [1]. In this study, we aim to: *Establish the protocol for measuring NKAc and apply it to breast cancer samples. Specifically, we tested different conditions of fluorescent dye concentration for labeling the target cells, incubation time, and the E:T ratio. Then, we preliminarily apply the established protocol for measuring NKAc of some breast cancer patients and then discuss the method to analyze the obtained data.*

MATERIALS AND METHODS

1. Materials

Peripheral blood was collected from breast cancer patients (hospitalized in the Military Hospital 103) and healthy controls (the medical staff of the Department of Immunology, Vietnam Military Medical University).

* *Exclusion criteria:* Donors were in status of acute infection or autoimmune diseases.

* *Location and time:* At the Department of Oncology, Military Hospital 103 and the Department of Immunology, Vietnam Military Medical University, from June to July 2024.

2. Methods

* *Study design:* To optimize the protocols, we used 4 blood samples from healthy medical staff. Then, apply the established protocol to the blood samples from 7 breast cancer patients and 5 healthy medical staff.

* *NKAc assay:*

Live fluorescent-CFSE-labelled K562 cells were killed when co-cultured with the NK cells (CFSE - 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester of CFDA SE, Biolegend, #423801). Dead K562 cells can be subsequently stained by dead-cell fluorescent dye (Zombie NIR, Biolegend, #423105) and counted by flow cytometry with a dual laser (red, blue laser). Heparinized

blood was collected from the donors. Peripheral blood mononuclear cell (PBMC) preparation was performed via the Ficoll-gradient separation method. The percentage of NK cells was analyzed via flow cytometry (described below). K562 cancer cell line was bought from Cell Line Service (CLS, Germany). Each condition was prepared in triplicate wells.

Flow cytometric analysis: Label the E:T suspension with the dead-cell fluorescent dye (Zombie NIR, Biolegend) following the manufacturer's recommendation in 15 minutes before analyzing on the flow cytometry analyzer (Novocyte, ACEA, USA). Analyzing and interpreting the data following Wu et al. (2020) [7]. Percent specific lysis [%] of K562 cells was calculated as the following method based on the percentage of dead K562 cells (CFSE+Zombie NIR+):

$$\text{Specific lysis [\%]} = [(n_1-n_2)/(100-n_2)]*100$$

n_1: Percentage of dead K562 cells in the E:T wells (Average of the triplicates); n_2: Percentage of dead K562 cells in the negative control wells (Average of the triplicates).

* *NKAs assay:*

NKAs or IFN-γ levels in the supernatant are measured via the ELISA method following the standard protocol of the IVD NK-VUE kit (ATGen, Korea).

* *NK cell subset analysis by flow cytometry:* PBMCs after Ficoll gradient separation were washed by PBS-1X and stained with a cocktail of fluorescent conjugating antibodies (Anti-CD45-PercP, anti-CD3-FITC, anti-CD56-PE from Biolegend). NK cells are defined as CD45+CD3-CD56+ by flow cytometry analysis.

* *NK cell purification*: To purify NK cells from peripheral blood, we followed the protocol of Phuc et al. (2023) [3] by using the Miltenyi Biotec human NK cell isolation kit.

* *Statistical analysis:* Data are presented as mean (standard deviation-SD). Statistical analysis was performed on Microsoft Excel (2023). T-test was used for the comparison of the two populations; $p < 0.05$ was considered to be statistically significant. Coefficient correlation (r) is interpreted as follows: 0.00 - 0.199: Very weak; 0.2 - 0.399: Weak; 0.4 - 0.599: Medium; 0.6 - 0.799: Strong; 0.80 - 1.00: Very strong.

3. Ethics

The study was approved by the Institutional Review Board of Vietnam Military Medical University (2352/QĐ-HVQY, June 2024). All donors were explained and consented to the study. The participants did not pay any fee related to the study. The Department of Oncology, Military Hospital 103, Vietnam Military Medical University granted permission for the use and

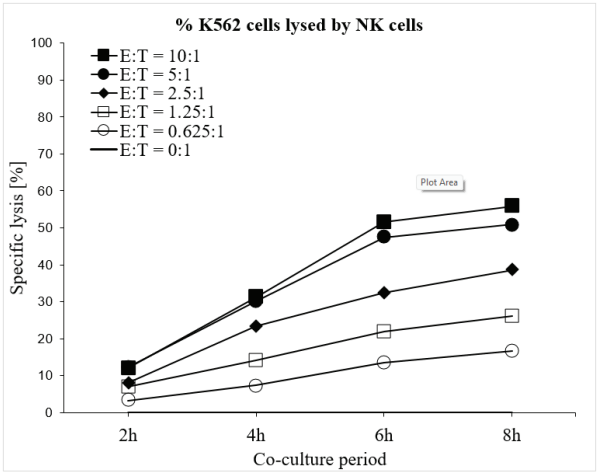
publication of the research data. The authors declare to have no conflicts of interest in the study.

RESULTS AND DISCUSSION

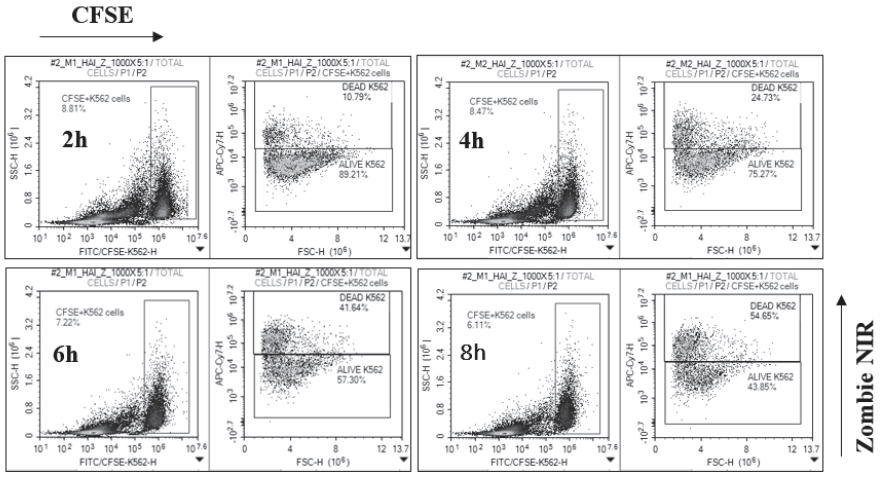
In an attempt to set up our own protocol to analyze NKAc, we have tested the following conditions. Firstly, we chose two fluorescent dyes (CFSE and Zombie NIR) to stain alive vs. dead target cells as they are read by the two distinct fluorescent channels (FITC by blue laser and APC-Cy7 by red laser), which means there is no overlapping signal between live cells (CFSE-FITC positive) and dead cells (Zombie NIR-APC-Cy7). In the procedure before co-culture, K562 cells would be stained by CFSE (conjugating to intra-cellular proteins). K562 cells stained with CFSE concentration > 2.5μM would show a strong and uniform population on flow cytometry (Median fluorescent intensity - MFI exceeded 10^6) (Data not shown). It is important to note that CFSE has been reported to lose its fluorescent signal during long incubation time [9]; thus, we thought it would be optimal to use 5μM of CFSE for pre-staining K562 cells. Next, we performed heat-killed CFSE-labelled K562 cells and stained them with dead-cell fluorescent dye (Zombie NIR - ZB). The dead cell population (CFSE+ZB+) could be clearly separated from the alive cell population (CFSE+ZB-) even when using the modest concentration of ZB (1:1000 dilution) as recommended by the manufacturer's suggestion (Data not shown). Thus we fixed the following condition of 5μM CFSE and 1:1000 diluted ZB concentration for staining alive and dead target cells when analyzing them by flow cytometry.

Most of the published protocols used fresh PBMCs instead of purified NK cells, and the reasons could be: (1) Isolating NK cells requires time & consumable costs and weakens the NK cell activity; (2) NK cells might be the major sole effector cells to kill K562 cells as it was shown that PBMCs without NK cells did not kill K562 cells [9]. However, since NK cells occupy a relative fluctuation frequency (5 - 15%) of PBMCs, fixing the number of PBMCs as the effector cells might be not a good approach. We always checked the frequency of NK cells (CD3-CD56+) on flow cytometry and adjusted the density of PBMCs based on the NK cell: K562 cell ratio. About the E:T ratio in terms of NK cells as the effector cells, we researched the previous publications and found that the E:T ratio has been used around 5:1 and 1:1 [1, 6, 9]. About incubation time, most papers showed a consensus of 4 hours [1, 6, 9]. In our established system, we checked again with a matrix of time (2 - 8 hours) and the E:T ratio (10:1 to 0.625:1). The data in figure 1

suggested that: (1) Incubation time should be 4 hours or > 2 hours to see a properly lysis activity of target cells; (2) There were no clear differences of NKAc between the two conditions of E:T = 10:1 and 5:1. Thus, we fixed the conditions of time and E:T ratio as 4 hours and 5:1, respectively.



(a)



(b)

Figure 1. NKAc varied depending on the parameters of co-culture time and E/T ratio.

(a) The specific lysis of K562 cells in the co-culture system with NK cells/PBMCs at different conditions of time and E:T ratios;

(b) A representative image of flow cytometric analysis of the co-culture system with the E:T ratio = 5:1 and different time incubation. The data are presented as the mean of 4 independent samples from healthy donors. E: Effector cells, T: Target cells.

Subsequently, we checked the stability of the NKAc assay by testing again the NKAc of 4 healthy donors with a time interval of 1 month. The average of NKAc (n = 4) remained unchanged with p > 0.05 (*Table 1*). Thus our protocol of the NKAc assay might be stable.

Table 1. NKAc of the donors at two timepoints (1-month interval).

Sample #	Specific lysis (%)	
	Timepoint #1	Timepoint #2
#1	14.3	20.5
#2	18.4	17.3
#3	24.0	28.4
#4	31.9	31.0
Mean	22.2	24.3
SD	7.6	6.5
p	> 0.05	

We then tried to apply the established assay to preliminarily examine a small number of donors, including some newly-diagnosed breast cancer patients (n = 7) and a healthy control group (n = 5). NKAc of the patients and the healthy control group were 26.0 ± 15.4% and 17.2 ± 8.7%; there was no significant difference with p > 0.05 (*Table 2*).

Table 2. NKAc in the study groups.

Specific lysis, % (Mean, SD)	
Breast cancer patients (n = 7)	26.0 (15.4)
Healthy controls (n = 5)	17.2 (8.7)
p	> 0.05

In order to preliminarily validate the optimized protocol, we tested the NKAc of the NK cells in PBMCs or the purified NK cells in the co-culture system with K562 cells with the defined E:T (NK cells: K562 cells). Data shown in table 3 suggested that the NKAc of NK cells in PBMCs could be similar to that of the purified NK cells.

Table 3. NKAc (% K562 cells) lysed by NK cells in PBMCs or purified NK cells.

E:T ratio (Mean ± SD)	NKAc (specific lysis, %)	
	PBMCs (NK cells)	Purified NK cells
E:T = 10:1	24.4 ± 2.3	35.6 ± 1.8
E:T = 5:1	20.9 ± 1.4	25.0 ± 1.7
E:T = 2.5:1	18.4 ± 0.2	18.9 ± 2.0
E:T = 1.25:1	13.5 ± 1.3	13.1 ± 1.1
E:T = 0.625:1	11.6 ± 0.7	9.7 ± 0.2
p	> 0.05	

Altogether, the study data suggest a favorable protocol that the NKAc assay could be performed by fixing the following criteria: (1) Staining CFSE with 5μM, Zombie NIR with 1:1000 dilution; (2) E:T co-culture in 4 hours with the E:T = 5:1; PBMCs density should be adjusted based on the frequency of NK cell in PBMCs.

Lastly, we discuss the NK cell activity in general when it comes to both NKAs and NKAc. NKAs were also documented in the 12 study participants. Our small-size data show that 3/7 breast cancer patients had NKAc levels < 500 pg/mL and 2/7 breast cancer had NKAs levels < 200 pg/mL; whereas we did not notice any NKAs of 5 healthy controls < 500 or 200 pg/mL. Interestingly, 2 breast cancer patients with NKAs levels < 200 pg/mL also showed the lowest NKAc of less than 10% (*Table 4*). There is a medium strength of correlation between NKAs and NKAc in this study (r = 0.523), though the sample size is small.

Table 4. NKAs and NKAc in the study groups.

Study groups Mean (SD)	NKAs (IFNγ, pg/mL)	NKAc (specific lysis, %)
Patients (n = 7)	1243 (1192)	26.0 (15.4)
Healthy controls (n = 5)	1505 (1042)	17.2 (8.7)
p	> 0.05	> 0.05
	r = 0.523	

In this study, we tried to establish an in-house protocol for examining NKAc of breast cancer patients based on the previously published characterization of non-standardized methods of assessing NKAc. Our study suggests a method that is rather feasible and stable to examine the NKAc of the patients.

However, as a preliminary study, our study has mainly focused on optimizing the protocol and has only been tested in small-size samples, which might hinder the strong conclusion of the study. Besides, we did not validate our established protocol with another gold-standard protocol. Thus, we did not give a reference range of NKAc; this task requires analyzing NKAc in larger-scale samples.

CONCLUSION

It is possible to assess the NKAc in breast cancer patients using the flow cytometric technique developed in this study. Additional research is required to confirm the diagnostic and prognostic significance of NK cell function tests.

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