VNU Journal of Science: Natural Sciences and Technology
Journal homepage: https://js.vnu.edu.vn/NST

Original Article

Immune Cells Expansion from Peripheral Blood of Some Vietnamese Cancer Patients

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Received 05 August 2021
Revised 20 August 2021; Accepted 25 August 2021

Abstract: Immunotherapy recently attracted high attention of scientists in cancer treatment. However, this therapy is poorly studied and applied in Vietnam. In this report, we present the results of immune cell ex vivo expansion for cancer treatment. After 20-21 days of culture, the average number of $CD3^+CD8^+$ cytotoxic T lymphocytes (CTLs) increased by 633.6-fold, equivalent to $3,277.5 \times 10^6$ cells, and with the cell viability of 96.3%. Among them, the increasing folds of lung, liver and gastric cancer patients were 232.4, 812.4 and 655.6, respectively. Meanwhile, the average number of $CD3^-$ $CD56^+$ natural killer (NK) cells increased by 940.3-fold, equivalent to $2,659.3 \times 10^6$ cells, with the cell viability of 95%. Gastric cancer patients had the highest-fold NK cell expansion (1,644.9-fold) compared to that in lung cancer patients (695.0-fold), and liver cancer patients (358.3-fold). These results revealed that our method of immune cell expansion met the requirements in the immune cell number for clinical applications in cancer treatment in Vietnam.

Keywords: Immune cell therapy, natural killer cells, cytotoxic T lymphocytes, peripheral blood mononuclear cells, cancer.

1. Introduction

Cancer is not only considered a leading cause of death but also affects population aging and growth in every country of the world. Cancer incidence and mortality are growing rapidly worldwide; there were estimated 19.3 million new cases and 10 million cancer deaths worldwide in 2020 [1]. Vietnam is undergoing a rapid socio-economic transition with an increasing cancer burden. According to Globocan in 2020, Vietnam had 182,563 new cancer cases, and 122,690 cancer deaths and the top 5 frequent cancers (excluding non-melanoma skin cancer) including liver cancer, lung cancer, breast cancer, stomach cancer, and colorectum cancer [1, 2]. The provision of high-quality medical services using advanced treatment

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https://doi.org/10.25073/2588-1140/vnunst.5291

methods is one of the efforts to reduce mortality from cancer, as well as improve the quality of life for cancer patients. This is an essential and urgent requirement, and among many cancer treatment methods, cancer immunotherapy is a noticeable method which attracts many scientists in recent years [1, 3].

Cancer immunotherapy is defined as a therapeutic approach that targets or manipulates the immune system to fight again cancer cells [4-7]. A method using autologous immune cells to strengthen the patient's immune system is called AEIT (Autologous Enhancement Immunological Therapy). The principle of this therapy is isolating immune cells from the patient's peripheral blood, then culturing them in order to expand their number and activate one or more cell types in specific conditions, and finally transfusing these expanded cells back into the patient's body [8, 9].

Natural killer (NK) cells are characterized by a lack of CD3 and the expression of CD56. The activity of NK cells depends on the balance between inhibitory receptors (KIRs) and activating receptors (NKRs) on their surface. The activating receptors recognize "induced-self" ligands - molecules that lack or are expressed at low levels on normal cells, but which are upregulated on unhealthy or cancer cells, while the inhibitor receptors recognize the Major Histocompatibility Complex (MHC) class I molecular which is present in almost all normal cells and down-regulated or lack on cancer cells. These help NK cells distinguish normal and abnormal cells in the body [10-13].

Cytotoxic T lymphocytes (CTLs) are characterized by the expression of both CD8 and CD3 on their surface. They have the ability to kill cancer cells that are NK cell-resistant. Besides, CTLs could also attack cancer cells through MHC-independent mechanism, killing cancer cells without affecting other cells in the body. CTLs are used for the treatment of multiple kinds of cancer including melanoma, hepatic cancer, lung cancer, gastric cancer,… [14-17]. The effectiveness and safety of therapy that uses autologous CTLs have been reported. Today, this therapy is applied broadly all over Japan and is recognized as an "advanced medical technology" by the Japanese Ministry of Health, Labor, and Welfare [17].

According to the above analysis, both NK cells and CTLs have the capacity of identifying and killing cancer cells through different mechanisms. NK cells detect target cells through MHC-I expression-dependent mechanism while the detection of CTL to target cell does not depend on MHC-I expression. One tumor could have several kinds of cancer cells with different antigen expressions so cancer cells in one tumor have heterologous properties. Therefore, the combination of two kinds of immune cells including NK cells and CTLs in AIET will bring more advantages in the detection and killing of cancer cells with or without MHC-I expression. There were many studies that demonstrated the effectiveness of the combination between these two kinds of immune cells on pre-clinical research models and clinical trials [11, 18, 19]. In these studies, the key factors for the success of the therapy were the sufficient number of activated immune cells. There are different methods to expand and activate NK cells and CTLs from the peripheral blood of cancer patients.

In this study, we presented the results of immune cells expansion from peripheral blood of nine patients belonging to three types of leading cancer in Vietnam: lung, liver and gastric cancers. These cells were expanded by using BINKIT developed by Dr. Terunuma (Biotherapy Institute in Japan), which was proved to efficiently induce the number of selective cells and activate them to kill cancer cells [8, 20, 21].

2. Methodology

2.1. Patients

Nine patients with confirmed diagnosis of gastric, liver or lung cancer and ECOG/PS (Eastern Cooperative Oncology Group performance status) $\geq$ 2 at the Oncology Department, Vinmec International Hospital, were enrolled in this study. The exclusion criteria were serious infection, autoimmune

diseases, using anti-rejection drug or T cell lymphomas. Patients signed the written consent which is approved by the Ethics Committee of Vinmec International Hospital. Nine peripheral blood samples were marked as PT1 to PT9 corresponding to 9 patients (PT). In patients who have undergone chemotherapy, if the peripheral blood white blood cell count is lower than normal, it needs to wait until the count rises above optimal levels for adequate NK cell and T cell sourcing and expansion.

2.2. Isolation and Large - Scale Expansion of Natural Killer Cells and Cytotoxic T Lymphocytes from Peripheral Blood

We already presented the method for immune cell expansion in [22]. Briefly, peripheral blood mononuclear cells (PBMNCs) were obtained by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Sweden) and were cultured using BINKIT® (Biotherapy Institute of Japan, Japan) at density of 1×10^6 cells/ml in cell initial Medium containing 0.01 KE/ml OK432 (Chugai Pharmaceutical, Japan), 700IU/ml recombinant human IL-2 (rhIL-2) (Chiron, Netherlands), supplemented with 5% of heat-inactivated autologous plasma, cultured in an anti-CD16 monoclonal antibody-immobilized culture flask for NK cell expansion. For CTLs expansion, PBMNCs were cultured in initial medium containing700IU/ml recombinant human IL-2 (rhIL-2) (Chiron, Netherlands) in ananti-CD3 monoclonal antibody-immobilized flask, and incubated under 5% of CO_2 at 37 ^oC for 3 days. After 3 days, the culture medium was changed and sub-cultured every 2-3 days in Subculture Medium containing 350 IU/ml recombinant human IL-2 (rhIL-2) (Chiron, Netherlands) supplemented with 5% of heat-inactivated autologous plasma to maintain the concentration of 0.8-1.0 $\times 10^6$ cells/ml, without discarding the old-medium. When the number of cells increased logarithmically, the cultured cells were transferred into culture bags (Nipro, Japan) until the end of the culture. The cell processing center was set up according to GMP standard.

2.3. Phenotypic Analysis

The phenotype of expanded cells and PBMNCs at baseline (day 0) and the end of culture were analyzed by flow cytometry. Monoclonal antibodies specific for CD3, CD8, CD56, CD4 conjugated with Pacific Blue, Fluorescein Isothiocyanate (FITC), R Phycoerythrin (PE) and Allophycocyanin-Alexa Flour 750 (APC-Alexa Flour 750) (Beckman Coulter, CA, USA), respectively; and the corresponding isotype were used for characterization of cell population. Cells were analyzed by Navios Cytometer (Beckman Coulter, USA) and data was acquired by Navios software, version 3.2 according to the manufacturer's instructions.

2.4. White Blood Cell Count

The complete blood cell count of patient's peripheral blood samples was performed using a Celltac Es MEK-7300K automated machine (Nihon Kohden, Japan) to determine the number of blood cell types such as red blood cells, white blood cells, and platelet.

2.5. Cell Count and Viability Testing

The viability of cell was conducted using Trypan Blue Stain 0.4% (Sigma Aldrich, USA, code T8154). Briefly, the sample was completely resuspended then mixed gently with dying solution in the ratio 1:1. A volume of 10 µl of mixed solution was applied in a chamber of a hemocytometer (Incyto C-Chip, Korea), then counted the unstained (viable) and stained (nonviable) cells separately in quadrants by a hand tally counter. The total number of viable cells (A) is calculated by the formula:

$$A = \frac{\text{Viable cell count}}{\text{Quadrants counted}} \times 2 \times 10^4 \text{ volume (ml)}$$

Cell viability is calculated as the number of viable cells divided by the total number of cells within the quadrants on the hemocytometer:

$$\text{Viability (\%)} = \frac{\text{Live cell counted}}{\text{Total cell counted}} \times 100\%$$

2.6. Statistical Analysis

Descriptive statistics were used to illustrate the patient characteristics and immune cell components. Quantitative variables were described by the mean value and their standard deviations, range.

A t-test was used to determine the relationship of blood cell component, immune cell over gender, age group while one-way ANOVA was used to assess the relationship of blood cell component, immune cell and cancer type. A p-value less than 0.05 was considered the threshold for significance. Data analyses were performed using STATA software version 12.

3. Results and Discussion

3.1. Patient Characteristics

Patients enrolled to this research between December 2015 and June 2017, included 8 males and 1 female, 3 patients for each of three cancer types: gastric, liver and lung cancers. The mean age of the participants was 66 ± 8.3 years (53-79 years old) (Table 1).

Table 1. Patient characteristics

Case	Age (years) at treatment	Gender	Cancer type
PT1	53	M	Gastric cancer
PT2	55	M	Gastric cancer
PT3	67	M	Gastric cancer
PT4	69	M	Liver cancer
PT5	79	M	Liver cancer
PT6	68	F	Liver cancer
PT7	77	M	Lung cancer
PT8	61	M	Lung cancer
PT9	65	M	Lung cancer

3.2. Immune Cell Expansion Ability

Peripheral blood of cancer patients was collected and cultured at the same day. At the collection, the mean number of white blood cells was $4.4\pm1.9 \times 10^6$/mL, among them the lymphocytes accounted for 26.2%±11.4%, and monocytes 7.0%±1.6%. The percentage of lymphocyte in mononuclear cells was 76.4%±10.4%. The isolated mononuclear cells were divided into two equal parts for NK cell expansion (NK cell culture) and CTLs expansion (CTLs culture). At the seeding of culture, the total number of cells was $38.5\pm20.2 \times 10^6$, NK cells accounted for 16.7% with absolute number of $4.5\pm3.4 \times 10^6$; CTLs accounted for 22.7% with the absolute number of $6.5\pm4.3 \times 10^6$ (Table 2).

After the first 3 days of culture, the number of cells decreased by 41.7% compared to the number at D0. Then the cell grew gradually, reached the logarithm phase from D11 of culture. There were two samples in each culture, that started to decrease in numbers from D17. In particular, one sample, PT7, stopped growing from D15 in CTLs culture. This sample also decreased in numbers at D17 in NK cell culture (Figure 1A). The phenotype of immune cells was determined based on surface markers expression. We determined these markers at D0 and the last day of culture. It was clearly different in the expression of CD3-CD56+ cell population in NK cell culture and CD3+CD8+ in CTLs culture (Figure 1B).

On days 20-21 of culture, the number and cell component in each culture condition had significant changes. In CTLs culture, the cells viability was 96.3% with the total number of cells was $5,273\pm2,848.4 \times 10^6$. Among them, CTLs cell number was $3,277.5\pm1,642.7 \times 10^6$ accounting for 64.2%, and increased more than 630-fold. Meanwhile, in NK cell culture, the cell viability was 95%. The total number of cells reached $3,087.6.0\pm2,252.6 \times 10^6$ with the number of NK cells was $2,659.3.1\pm1,690.8 \times 10^6$ accounting for 88% of cells population. The expansion of NK cells was impressively high of a 940-fold increase compared to the number of NK cells at seeding (Figure 2).

Table 2. The immune cell components at the blood collection day

Immune cell component		Mean±SD	[Min, Max]
At blood collection	*WBC (10^6/ml)*	4.4±1.9	[3.2, 10]
	% Lym	26.2±11.4	[8.8, 39.9]
	% Mono	7.0±1.6	[3.9, 8.2]
	% Lym/MNC	76.4±10.4	[54.3, 90.8]
At cell seeding	*Total cell seeding ($\times 10^6$)*	38.5±20.2	[23.0, 79.0]
	% NK	16.7±8.4	[2.5, 30.1]
	Absolute number of NK ($\times 10^6$)	4.5±3.4	[1.5, 13.4]
	% CTLs	22.7±6.3	[9.9, 30.7]
	Absolute number of CTLs ($\times 10^6$)	6.5±4.3	[3.3, 18.3]

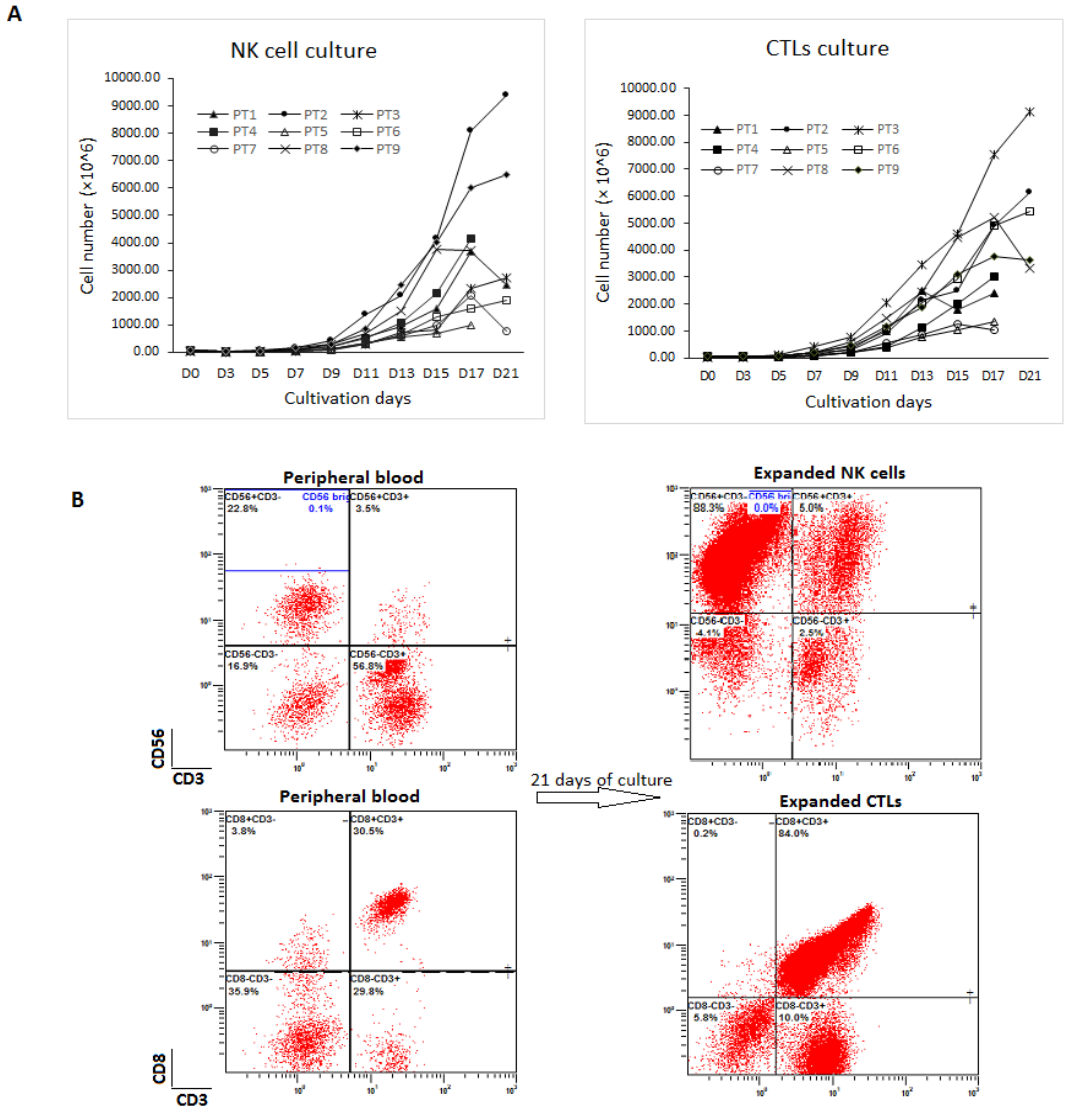

Figure 1. Cell growth curves in NK cell culture and CTLs cell culture (A). Phenotyping analysis of immune cell population at D0 and D21 of culture. D: day of culture.

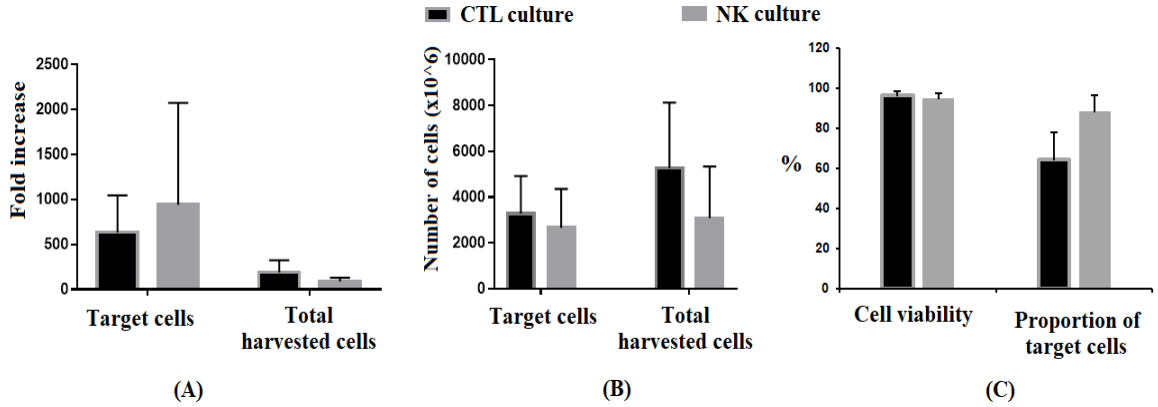

Figure 2. Immune cell expansion ability in CTL culture and NK cell culture. (A) The fold increase in target cells and total cells. (B) The average number of target cells and total harvested cells. (C) The cell viaility and proportion of target cells. The target cells are CTLs and NK cells in TL culture and NK cell culture, respectively.

Autologous immune enhancement therapy (AIET) has been widely used in the last two decades for cancer treatment. This method was proved to be safe and contribute to increasing by 30% of efficacy in cancer treatment when using with other traditional methods such as surgery, chemotherapy, or radio therapy [20]. The key point for the successful application of this method is the adequate number of immune cells expanded from a very little number of them in the peripheral blood of cancer patients [21].

In most of the published cases, the doses of cells were diverse depending on the expansion method or the status of patients. Kananathan et al. reported a rare case of advanced epithelioid sarcoma used AIET with 7 infusions containing the average numbers of NK cells and CTLs cells per dose were 1,880 $\times 10^6$ and 1,760 $\times 10^6$, respectively, which were more than 100-fold expansion compared to the initial seeding number. This patient had an overall survival of 25 months without any further chemotherapy [23]. In another case of locally advanced carcinoma of the cervix, the average numbers of expanded NK cells and CTLs cells were 282.5 x 10^6 and 478.5 x 10^6, respectively. After six transfusions, there was a complete resolution of retroperitoneal lymph node with no evidence of local lesion of the cervix [24]. A case report of a stage IV colonic cancer showed the average expansion folds of NK cells and CTLs cells were 44 and 168, respectively [25]. AIET has been used not only for solid tumors, but also for liquid tumors. A case report of a 15-year-old girl with Philadelphia Chromosome Positive Acute Lymphoblastic Leukemia indicated that the AIET was efficient in erasing cancer cells when used together with chemotherapy and the remission continued 5 years after treatment. The average number of NK cell expansion was 562 × 10^6 [26].

Compared to these publications, our results showed that we had successfully expanded NK cells and CTLs cells with the higher average numbers of 2,659.3 x 10^6 and 3,277.5 x 10^6, respectively. Moreover, the frequencies of selective cells in each expanded population were rather high compared to the previously reported study.

3.3. The Relative Relationship of Immune Cell Expansion Ability to the Patient's Age, Gender and Cancer Type

The proportion of monocytes of the patients aged below 65 was significantly lower than that of the people aged above 65 ($p<0.001$), while the ratio of lymphocytes to monocytes of

65-and-older group was 87.4±4.5%, which was higher than 73.6±9.6% of under age 65 group (p=0.0335). However, the average density of white blood cells was the highest in lung cancer patients, followed by liver cancer patients and gastric cancer patients (p=0.008) (Table 3).

Table 3. The relative relationship of blood cell components at blood collection time to the patient's age, and cancer types

	White blood cells (10^6/ml) (Mean± SD)	% Lymphocyte (Mean± SD)	% Monocyte (Mean± SD)	% Lym/MNC (Mean± SD)
Age (years old)	p=0.7941	p=0.3706	p=0.000*	p=0.0335*
≤ 65	4.1±0.3	31.7±9.8	4.2±0.3	87.4±4.5
> 65	4.4±2.1	24.8±11.7	7.6±0.9	73.6±9.6
Cancer type	p=0.008*	p=0.094	p=0.2075	p=0.097
Gastric	3.4±0.5	39.0±1.6	6.9±2.1	85.3±3.7
Liver	4.3±1.5	17.9±4.4	7.7±0.2	68.8±7.1
Lung	6.4±3.2	17.1±5.4	5.7±1.8	73.8±13.2
**p<0.05*				

At the cell seeding of culture, the percentage of NK cells was significant lower in the age group below 65 to the group above 65 (7.1% vs. 19.1%, p=0.0205). Moreover, the number of NK cells at the starting day of culture was highest in lung cancer patients (7.3 million cells) compare to the liver cancer (5.4 million) and gastric cancer (2.3 million), with p=0.002 (Table 4).

After 20-21 days, in both CTLs and NK cell culture, there was no significant differences in the cell viability, percentage of CTLs/NK cells, and the total number of cells between ages, and cancer types (p>0.05) (Table 5, 6).

Table 4. The relative relationship of immune cell components at cell seeding point to the patient's age, and cancer types

	Number of cells at seeding ($\times 10^6$) (Mean± SD)	% NK cell (Mean± SD)	Number of NK cells (Mean± SD)	% CTLs (Mean± SD)	Number of CTLs (Mean± SD)
Age (years old)	p=0.9888	p=0.0205*	p=0.2251	p=0.6843	p=0.1024
≤ 65	44.7±18.3	7.1±4.1	2.3±0.5	24.1±5.8	10.1±7.1
> 65	37.0 ±21.1	19.1±7.5	5.1±3.6	22.3±6.6	5.6±3.1
Cancer type	p= 0.588	p=0.089	p=0.002*	p=0.329	p=0.085
Gastric	34.0±16.0	9.3±3.3	2.3±0.6	27.1±3.2	8.1±5.0
Liver	39.7±26.1	22.0±5.0	5.4±2.8	20.0±6.5	5.3±4.6
Lung	45.2±19.3	21.0±11.5	7.3±5.7	19.2±6.6	5.7±0.7
**p<0.05*					

Table 5. The relative relationship of immune cell components in CTLs cell culture at cell harvesting to the patient's age, and cancer types

	Cell viability (%) (Mean± SD)	Number of viable cells ($\times 10^6$) (Mean± SD)	% CTLs (Mean± SD)	Number of CTLs cells ($\times 10^6$) (Mean± SD)	Total number of cells ($\times 10^6$) (Mean± SD)
Age (years old)	p=0.8758	p=0.3924	p=0.1158	p=0.8054	p=0.399
≤ 65	96.1±1.7	3962±1938.4	75.5±7.2	3056.7±1751.3	4122.9±1997.0
> 65	96.3±2.4	5601.3±3009.5	61.4±13.8	3332.7±1691.3	5768.6±3062.3
Cancer type	p= 0.056	p=0.481	p=0.197	p=0.801	p=0.468
Gastric	97.4±0.6	7499±2784.7	63.3±12.7	4497.9±1302.2	7705.3±2861.0
Liver	96.8±1.5	4347.3±1769.7	70.2±7.9	3032.3±1201.7	4468.8±1780.4
Lung	92.9±2.7	2673.3±1412.8	54.2±22.8	1327.2±828.5	2849.3±1460.9

Table 6. The relative relationship of immune cell components in NK cell culture at cell harvesting to the patient's age, gender and cancer types

	Cell viability (%) (Mean± SD)	Number of viable cells ($\times 10^6$) (Mean± SD)	% NK (Mean± SD)	Number of NK cells ($\times 10^6$) (Mean± SD)	Total number of cells ($\times 10^6$) (Mean± SD)
Age (years old)	p=0.2962	p=0.0695	p=0.4275	p=0.0734	p=0.0759
≤ 65	93.7±0.9	5181±3687.4	85.0±10.7	4212.0±2533.2	5507.8±3865.2
> 65	95.3±2.5	2564.3±1578.6	89.1±7.3	2271.1±1284.5	2724.0±1787.4
Cancer type	p= 0.083	p=0.140	p=0.019	p=0.256	p=0.131
Gastric	95.2±1.1	3771.9±2815.6	91.1±9.5	3255.6±1944.3	3970.8±2978.4
Liver	96.5±0.8	2115.5±1062.8	88.2±2.2	1858.9±934.2	2199.8±1130.8
Lung	91.4±2.7	3663.2±2853.2	83.1±11.2	3067.6±2267.2	4062.6±3253.6

However, there was a significant difference in the number of selective cells in each culture condition. In the NK cell culture, the fold increase in the number of NK cells in the age below 65 years was significant, 3.7 times higher than the age above 65 (p=0.0138). Especially, the expansion of NK cells was the highest at gastric cancer (1.6 billion cells) compared to lung cancer (0.695 billion cells) and liver cancer (0.358 billion cells) ($p<0.05$) (Table 7).

A study of Remarque E (1998) reported that the decrease in the NK cell number was associated with the increase in the risk of mortality rate in elderly people [27]. This immune cell type also plays fewer functions in vitro when isolated from elderly donors with a significant decrease in the cytotoxicity and the capacity of proliferation [28]. Similarly, Gounder et al., showed that the proliferation rate of NK cells upon aging declines when compared to the young age group [29].

Our results were also consistent with these results. It indicated that the immune cell expansion ability had a correlation to the age of the patients but not the initial number of cultured cells. Particularly, the expansion ability of NK cells was significantly different between the three types of cancer, suggesting that the biology of cancer type could have different impact on NK cell proliferation. However, the number of patients in this study is small with only 3 cases per each type of cancer. Therefore, in the perspective work, we will study on a statistical number of patients to overcome this limitation.

Table 7. The relative relationship of immune cell expansion ability to the patient's age, and cancer types

	Fold increase in total cell count (Mean± SD)		Fold increase in the number of selective cultured cells (Mean± SD)		Fold increase in the percentage of selective cultured cells (Mean± SD)	
	CTLs	NK	CTLs	NK	CTLs	NK
Age (years old)	p=0.1646	p=0.1962	p=0.1463	p=0.0138*	p=0.8042	p=0.0071*
≤ 65	91.3±18.1	115.3±38.0	320.5±72.8	2295.2±2099.3	3.2 ±0.5	16.5±11.6
>65	213.9±140.0	82.3±37.4	711.9±425.5	601.6±452.5	3.0±1.3	5.7±2.8
Cancer type	p= 0.228	p= 0.598	p=0.219	p=0.000*	p=0.144	p=0.014*
Gastric	269.0±144.8	110.4±34.8	655.6±292.1	1644.9±1510.7	2.3±0.7	12.3±8.5
Liver	171.7±108.5	69.6±29.0	812.4±494.2	358.3±94.7	3.8±1.5	4.3±1.7
Lung	65.6±37.3	84.5±52.7	232.4±149.4	695.0±807.3	2.8±0.5	5.8±5.2
**p<0.05*						

4. Conclusion

In the study, we successfully expanded the immune cells from peripheral blood of gastric, liver and lung cancer patients. The number of expanded cells met the requirement for clinical use. This is an important step to further applying AIET in cancer treatment in Vietnam.

Acknowledgements

This study was financially supported by Vingroup JSC.

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