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Polyfunctionality of Natural Killer Cell in Healthy Donors

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Abstract

Background: Natural killer (NK) cells are important guards of the innate immune system, which act by performing as primary effector cells in viral infections. NK cell function is regulated by the engagement of activating and/or inhibitory receptors on individual NK cell surfaces. Subsequent to activation, the release of preformed cytolytic granules or cytokines occurs. Recently, the polyfunctionality of NK cells has been described as a potent NK cell subset that mediates antiviral response in HIV-infected slow progressors. **Objectives:** To evaluate the polyfunctional NK cells in healthy individuals. **Methods:** Peripheral blood mononuclear cells (PBMCs) were separated from 41 healthy blood donors by Ficoll-Hypaque gradient centrifugation. Multicolor flow cytometry was used to investigate the expression of function markers (degranulation marker (CD107a), IFN-γ, and TNF-α) on NK cells following PMA/Ionomycin or K562 stimulation. **Results:** The percentage of NK cells expressing CD107a, IFN-γ, or TNF-α in response to PMA/Ionomycin were 17.85, 10.56, and 2.66 %, respectively. The NK cells expressing CD107a, IFN-γ, or TNF-α in response to K562 stimulation were 6.43, 2.09, and 0.57 %, respectively. The capability of NK cells to perform polyfunctions was 6.19 % of the total NK cells following PMA/Ionomycin stimulation, while 1.06 % was observed following K562 stimulation. The trifunctional $CD107a^{+}$ / $IFN\text{-}\gamma^{+}$ / TNF-α^{+} NK cell subset was found to be 0.95 and 0.04 % following PMA/Ionomycin and K562 stimulation, respectively. **Conclusion:** A small fraction of NK cells was capable of performing polyfunctions following stimulation, with less than 1 % being able to perform trifunctions in this study setting.

Keywords: Polyfunction, NK cells, healthy donor, innate immunity

Introduction

Natural killer (NK) cells are large granular lymphocytes derived from bone-marrow. NK cells comprise 10 - 15 % of peripheral blood lymphocytes, and play a role in innate immune responses for the removal of tumors or viral transformed cells without prior sensitization [1]. NK cells are activated by cytokines or interacted with target cell expressing ligands for NK cell receptors [2]. The outcome of NK cells activity is regulated by 2 type receptors, inhibitory and activating. Some inhibitory receptors of NK cells recognize self-MHC class I presented on almost completely healthy cell to prevent NK cell attacking [2]. Also, activating receptors bind to host-derived or pathogen encoded ligands which up-regulate on stress or infected cells. The integration of signals from both activating and inhibitory receptors triggers two main NK cells effector functions. First, cytotoxicity NK cells directly lyse target cells by releasing preformed cytolytic granules which contain perforin and granzyme [1]. The other secretes cytokines and chemokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Besides the function of the primary effector, NK cells have the ability to prime the adaptive immune response by secreting cytokines involved in the maturation of dendritic cells (DCs) [3].

In general, the population of NK cells is distinguished by the surface expression of CD56 and the lacking of CD3. The 2 major subpopulations of NK cells are divided by density of CD56 and CD16 surface expression. The major population of NK cell has a low density of CD56 expression, $CD56^{dim}$, and a high level of FcγIII receptors, $CD16^{+}$. This subpopulation mediates cytolytic activity via ADCC and releases cytokines, chemokines, and cytotoxic granules. The minor subpopulation has a high density of CD56 expression, $CD56^{bright}$, but a low or no expression of CD16. $CD56^{bright}$ $CD16^{+/-}$ NK cells have less cytotoxicity but produce more pro-inflammatory cytokines.

Unlike T and B cells, NK cells do not rearrange gene encoding receptors for specific recognition. NK cells are a heterogeneous population composed of various expressions of activating and inhibitory receptors. NK cells can modulate their activity by altering their receptor expressions during infection [4]. The alteration of NK cell subsets and functions has been reported. The expansion of $CD56^{bright}$ NK cells and the higher frequency of IFN-γ production in this NK cell subset after stimulation with Phorbol myristate acetate (PMA) and Ionomycin were observed in chronic HCV infected individuals in comparison to spontaneous resolved individuals [5]. Additionally, $CD56^{-}$ $CD16^{+}$ NK cells, a highly dysfunctional NK cell subset, were increased in HIV-infected viremic individuals [6,7]. Evidence in T cells suggested that polyfunctional T cells were associated with an increase in protective immune responses, particularly the breadth of cytokine secreted against pathogen or antigen exposure [8-10]. Similarly, trifunctional potential ($CD107a^{+}$ TNF-α^{+} IFN-γ^{+}) NK cells have been described as a potent NK cell subset mediated antiviral response in the early stage of HIV infected slow progressors [11].

The study of NK cell function in the Thai population in comparison to the Northern American population in the context of HIV infection has been reported [12]. Although the number and percentage of NK cells were similar between Thai and North American subjects, the cytolytic activity of NK cell was significantly higher in Thai subjects. Moreover, NK cells from HIV seropositive Thai subjects showed higher NK cell numbers and functions than HIV seropositive North Americans. The polyfunctionality of NK cells has been studied in Canadian and the United State populations [11,13]. However, polyfunctional NK cells are not well described in the Thai population. In this study, we aimed to evaluate the polyfunctional NK cells in healthy Thai individuals by using multi-parameter flow cytometry analysis. After stimulating cells with PMA/Ionomycin or K562 cells, the function of NK cells were characterized by measuring the expression of a degranulation marker, CD107a, and the production of TNF-α and IFN-γ. The reference value of this study could serve as a baseline for the further investigation of NK cell function in diseases.

Materials and methods

Study subjects

ACD-Whole blood was obtained from 41 healthy blood donors from the Blood Bank Section of Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Thailand. This study was approved by the ethics committee of the Faculty of Medicine, Chiang Mai University (No.083/2011). Each subject was informed and asked to sign a consent form.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Ficoll-Hypaque (AXIS-SHIELD). The residual red blood cells were hemolyzed with hypotonic lysing solution (NH_4Cl). Separated cells were washed with sterile PBS and RPMI1640 medium (Gibco), prior to freezing in fetal calf serum (FCS) (Gibco) containing 10 % (v/v) DMSO (Sigma-Aldrich) as cryopreserved PBMCs, until analysis.

Quantification of degranulating and cytokine secreting NK cells by flow cytometry

Cryopreserved PBMCs were thawed in RPMI1640 containing 20 % FCS or RPMI-20 and washed with RPMI1640 medium. PBMCs were resuspended at a final concentration of 5×10^{6} cells/mL in warm 10 % FCS in RPMI (complete RPMI: cRPMI) in the presence of 100 unit/ml recombinant interleukin 2

(rIL-2) (PeproTech). Finally, PBMCs were rested in 95 % humidified, 5 % CO_2 incubator at 37 °C overnight (16 - 18 h).

IL-2 stimulating PBMCs were washed with RPMI medium and adjusted to 1×10^6 cells in cRPMI. For NK cell functional study, PBMCs were stimulated with 2.5 μg/mL of phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) and 0.5 μg/mL of Ionomycin (Sigma-Aldrich), as non-specific stimulation. The HLA-devoid cell line, K562, was co-cultured with PBMCs in an optimized ratio of Effector to Target as 10:1 (data not shown), as receptor-ligand stimulation. The fluorescein isothyocyanate (FITC) labeled anti-CD107a (H4A3; BD Biosciences) were added into each condition as an extracellular marker of degranulation. Then, they were incubated at 37 °C in a 95 % humidified, 5 % CO_2 incubator. After 1 h of incubation, brefeldin A (final concentration at 10 μg/mL) (Sigma-Aldrich) and monensin (final concentration at 2mM/10^6 cells) (Sigma-Aldrich) were added in order to block golgi exportation [14] and intracellular protein transportation, and all were incubated for another 5 h.

Recombinant IL-2 activated cells were stained with a monoclonal antibody panel for phenotypic study, including ECD-conjugated CD3 (UCHTI; Beckman Coulter), Krome orange-conjugated CD19 (J3-119; Beckman Coulter), PerCP-conjugated CD14 (MϕP9; BD Biosciences), APC conjugated-CD56 (N901; Beckman Coulter), and Pacific blue-conjugated CD16 (3G8; BD Biosciences) for 30 min at 4 °C in dark conditions.

For intracellular staining, NK cells were fixed and permeabilized according to standard intracellular staining protocols for flow cytometry [14,15]. First, cells were fixed with 2 % paraformaldehyde in PBS for 10 min at room temperature. FACs wash buffer was added to stop reaction. NK cells were then stained and permeabilized at 4 °C with 0.5 % saponin in PBS, together with Alexa Flour 700-labeled anti-IFN-γ (B27; BD Biosciences) and PE-Cy7 labeled anti-TNF-α (Mab11; BD Biosciences) for 30 min, in dark conditions. Phenotypes and functions of NK cells were analyzed by a flow cytometer. Viable cells were gated according to their typical forward/side scatter characteristics, and at least 200,000 lymphocyte relevant events were acquired. The acquired flow cytometric data was analyzed by using Kaluza. The total NK cells were analyzed from CD3, CD14, and CD19 negative populations. Data was demonstrated as a median percent of population (range).

Statistical analysis

A student t-test was used to determine the difference between the expression of each surface marker and the number of NK cell functional responses following stimulation with the HLA devoid cell line, K562, and PMA/Ionomycin. Statistical significance was assumed at $p < 0.05$. The SPSS program was applied to assess the statistical significance of the difference between sample groups.

Results and discussion

Characteristics of study population

Forty-one healthy donors were recruited for this study (**Table 1**). Male gender comprised 80 % (n = 33) of the study population, and female comprised 20 % (n = 8). The median age was 34 years old, which ranged between 19 - 55 years. The median white blood cell count (WBC) and % lymphocyte within range were 9,413 (5,665 - 18,893) cells/μL and 25.2 % (12.1 - 43.4 %), respectively.

Table 1 Characteristic of study population.

Variable	Healthy donor (*N* = 41)
Gender; Male	33 (80 %)
Female	8 (20 %)
Age, year	34 (19 - 55)
White blood cell count, x 10^6cells/μL	9,413 (5,665 - 18,893)
Lymphocyte count, %	25.2 (12.1 - 43.4)

Data was presented as the median (range) of individuals in this study. *N*: number of subjects in group.

The number of NK cell subsets

The strategy of NK cell gating is illustrated in **Figure 1**. The NK cell population was set on negative expressions of CD3, CD14, and CD19. The subpopulations of NK cells were determined by surface expressions of CD56 and CD16 (**Figure 2A**). The median percentage of each NK cell subset from 41 healthy donors is shown in **Figure 2B**. The majority NK cell subset, $CD56^{dim}CD16^{+}$ NK cell contributed to 95.0 % (69.1 - 98.8 %) of the total NK cells. The $CD56^{bright}CD16^{+/-}$ and $CD56^{neg}CD16^{+}$ NK cell subset were found to be 3.0 % (0.4 - 25.0 %) and 1.8 % (0.4 - 14.7 %), respectively.

Degranulation and cytokine production of NK cells

The analysis of NK cell function is shown in **Figure 3**. The degranulation function of NK cells was studied using CD107a as a degranulation marker in the absence and presence of PMA/Ionomycin or K562 stimulation. After 6 h incubation, the expression of CD107a was detected in 17.85 % (range 0.92 % - 54.99 %) of NK cells following PMA/Ionomycin stimulation, and 6.43 % (range 0.33 - 46.16 %) following K562 stimulation. In a simultaneous experiment, the production of TNF-α and IFN-γ was analyzed by intracellular staining. After stimulation with PMA/Ionomycin or K562, TNF-α producing cells were 2.66 % (range 0.11 - 17.68 %) and 0.57 % (range 0.04 - 6.15 %), respectively. IFN-γ producing cells were 10.56 % (range 0.63 - 36.62 %) and 2.09 % (range 0.02 - 27.69 %).

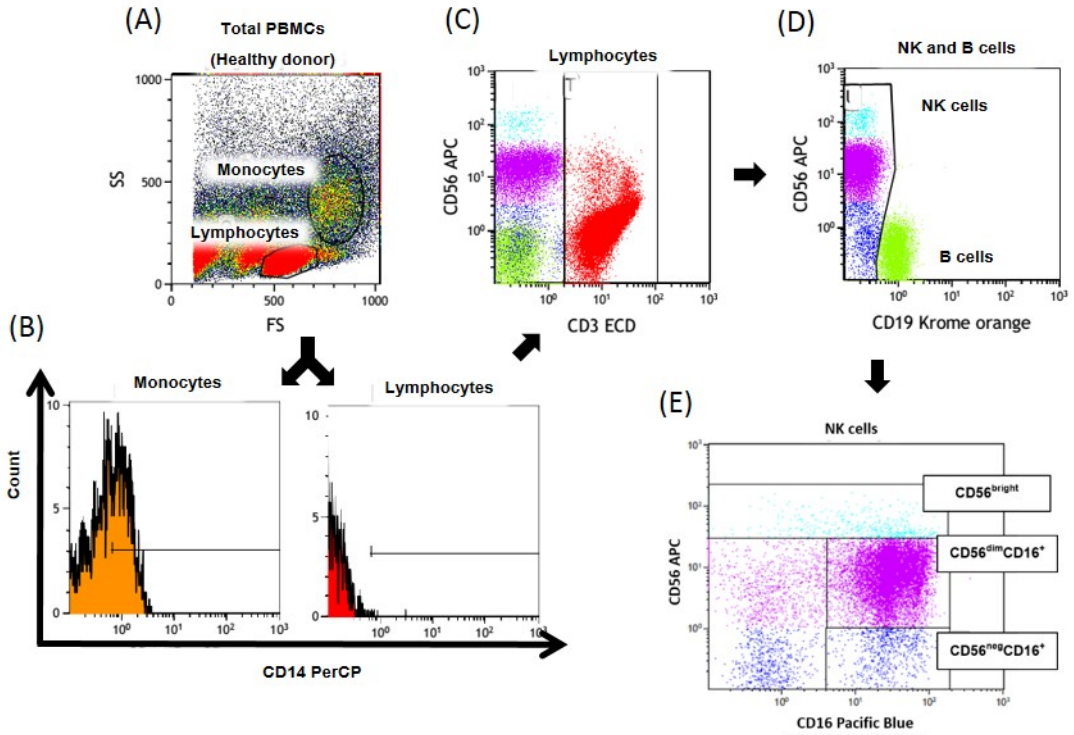

Figure 1 Strategy of NK cell gating from cryopreserved PBMCs. Cryopreserved PBMCs were stained with CD3, CD14, CD19, CD56, and CD16 antibodies conjugated to 5 different fluorochromes. From the light scatter (A), lymphocyte population was gated. NK cells were identified by negative population of CD14 (Monocytes) (B), CD3 (T lymphocytes) (C), and CD19 (B lymphocytes) (D). $CD3^{-}CD14^{-}CD19^{-}$ NK cells were determined as total NK cells (E).

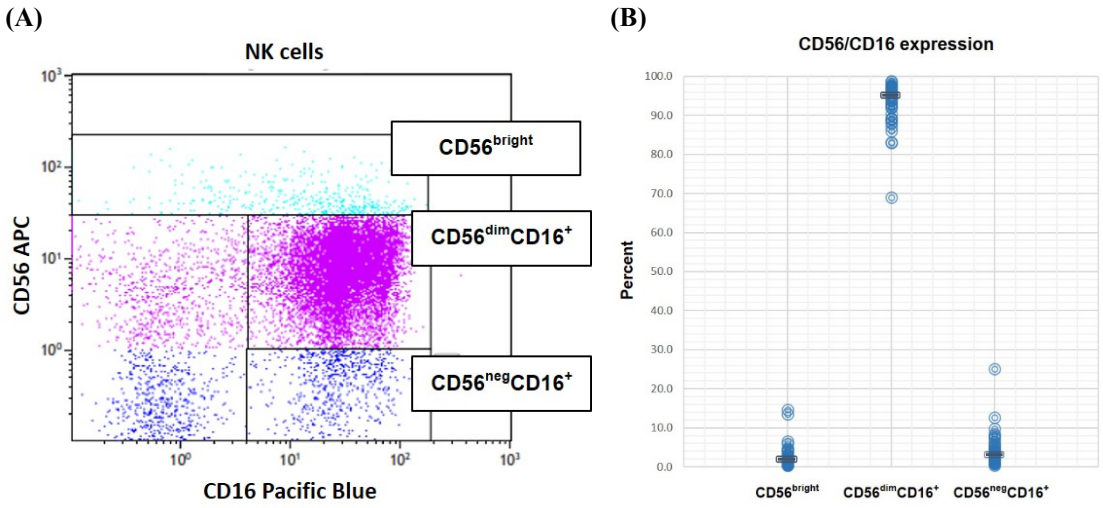

Figure 2 Phenotypic of normal NK cells. Total NK cells were characterized for CD56 and CD16 surface expression. (A) Dot plot from flow cytometer represents the distribution of NK cell expressing CD56 (y-axis) and CD16 (x-axis) of healthy donor individuals. (B) Scatter plot represent distribution percentage in three subsets of CD56/CD16 NK cell subpopulation. The bar graphs represented the median of each subset.

Polyfunctional NK cells in healthy individuals

To investigate the polyfunctionality of NK cells, the number of NK cell expressing CD107a and producing TNF-α and IFN-γ in response to PMA/Ionomycin and K562 stimulation were analyzed simultaneously. The measurement of 3 effector functions provided 7 possible NK cell function profiles. The median percentage of positive NK cells in each functional profile to total $CD3^-$ $CD14^-$ $CD19^-$ NK cells are illustrated (**Figure 4**).

The percentage of NK cells that expressed only CD107a or produced only TNF-α and IFN- γ in response to PMA/Ionomycin stimulation were 12.69 % (1.55 - 40.97 %), 0.67 % (0.12 - 4.63 %), and 6.44 % (0.87 - 15.60 %), respectively. NK cells expressing combinations of $CD107a^+$/$TNF\text{-}\alpha^+$, $CD107a^+$/$IFN\text{-}\gamma^+$, and $TNF\text{-}\alpha^+$/$IFN\text{-}\gamma^+$ subsets have been detected in 0.20 % (0.01 - 3.69 %), 3.95 % (0.50 - 20.86 %), and 1.09 % (0.05 - 5.06 %), respectively. The median percentage of PMA/Ionomycin stimulated NK cell subsets that simultaneously express 3 markers of NK cell functions was 0.95 % (0.05 - 17.72 %). Following K562 stimulation, the percentage of monofunctional NK cell subsets expressing CD107a, TNF-α, and IFN- γ were 6.11 % (0.50 - 31.10 %), 0.28 % (0.04 - 1.65 %), and 0.90 % (0.09 - 19.78 %), respectively. Combinations of $CD107a^+$/$TNF\text{-}\alpha^+$, $CD107a^+$/$IFN\text{-}\gamma^+$, and $TNF\text{-}\alpha^+$/$IFN\text{-}\gamma^+$ NK cell subsets have been detected in 0.05 % (0.01 - 0.78 %), 0.95 % (0.01 - 7.23 %), and 0.02 % (0.01 - 0.18 %), respectively. The trifunctional NK cell subset can be detected in as few as 0.04 % (0.01 - 1.31 %). Indeed, the percentage of NK cells in response to PMA/Ionomycin stimulation was higher than K562 stimulation in all functional profiles.

To address the contribution of functional NK cells following different stimulations, the percent contribution of each functional subset was calculated using the number of positive cells in each profile per total functional marker positive NK cells. The percent contribution of each functional profile is shown in **Figure 5**. We found that the predominant function of NK cells was cytolytic activity, as shown by the CD107a expression. Of the total functional markers of positive NK cells, 47.82 % (range 11.74 - 84.69 %) and 66.88 % (range 34.63 - 95.96 %) expressed CD107a alone after PMA/Ionomycin and K562 stimulation, respectively. Regarding cytokine production, the number of $IFN\text{-}\gamma^+$ NK cells was higher than $TNF\text{-}\alpha^+$ NK cells. Following PMA/Ionomycin stimulation, 25.11 % (range 3.69 - 59.90 %) and 2.56 % (range 0.48 - 16.29 %) of functional NK cells produced IFN-γ and TNF-α, respectively. Similarly, 13.47

% (range 0.82 - 45.50 %) and 3.35 % (range 0.20 - 25.08 %) of functional NK cells produced IFN-γ and TNF-α after K562 stimulation, respectively. The proportion of polyfunctional NK cells was 20.23 % following PMA/Ionomycin stimulation. $CD107a^{+}$/ $IFN\text{-}\gamma^{+}$ NK cells were found in the highest proportion (12.37 %, 4.47 - 32.01 %). Similar results were observed when NK cells were stimulated with K562. The proportion of polyfunctional NK cells was 10.81 % following stimulation. Bifunctional NK cells, $CD107a^{+}$/ $IFN\text{-}\gamma^{+}$, were also found in the highest proportion (9.66 %, 0.11 - 28.21 %).

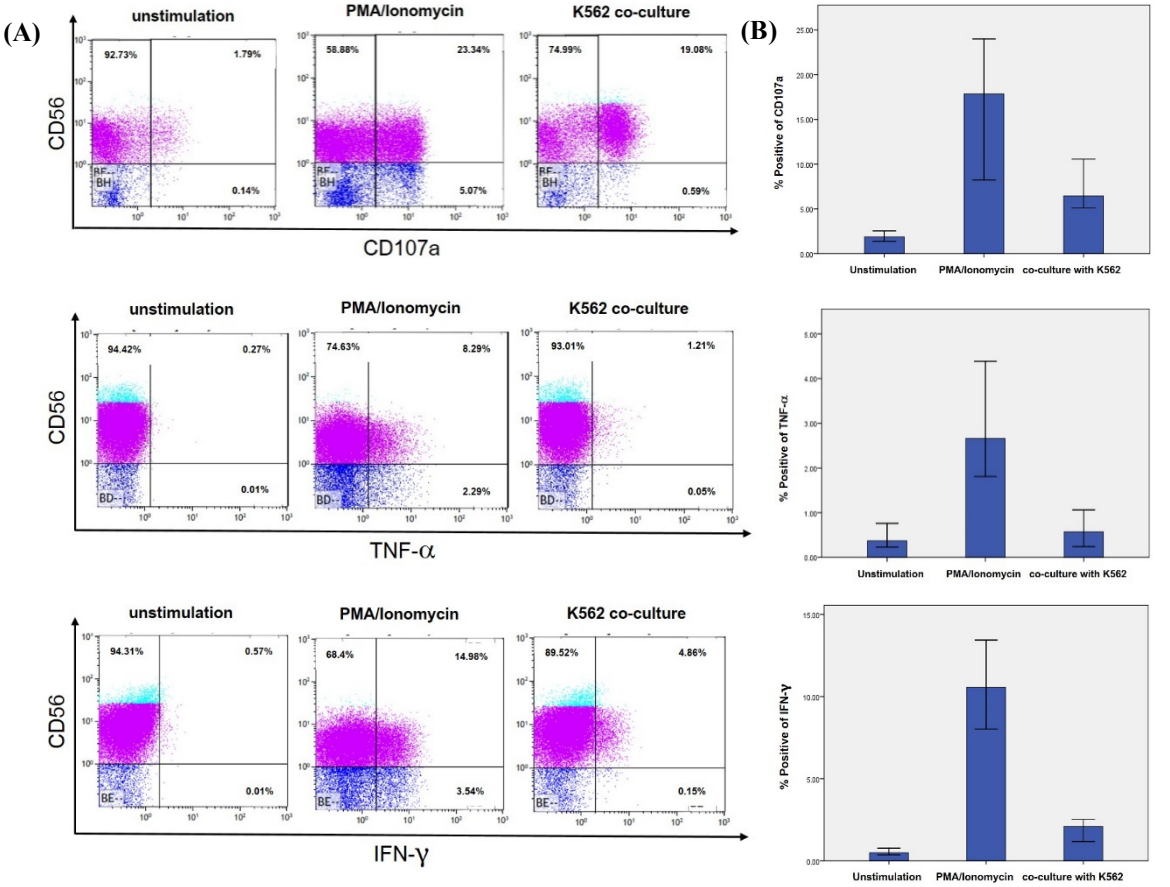

Figure 3 NK cells expressed CD107a and produced TNF-α and IFN-γ. PBMCs were stimulated with PMA/Ionomycin or K562 cell line and stained with fluorochrome labeled monoclonal antibodies to cell surface phenotypic markers and effector molecules. Samples were analyzed by flow cytometry and NK cells were gated ($CD3^{-}CD56^{+}$lymphocytes). After that, the surface expression of CD107a (as a marker of degranulation) and the presence of intracellular TNF-α and IFN-γ were evaluated in the NK cell population. (A) Dot plots of CD107a, TNF-α or IFN-γ expressing NK cells in unstimulated, PMA/Ionomycin stimulated, and K562 stimulated conditions are shown. (B) The median percentage of NK cells expressing CD107a, TNF-α and IFN- γ are demonstrated in bar graphs with 95 % CI.

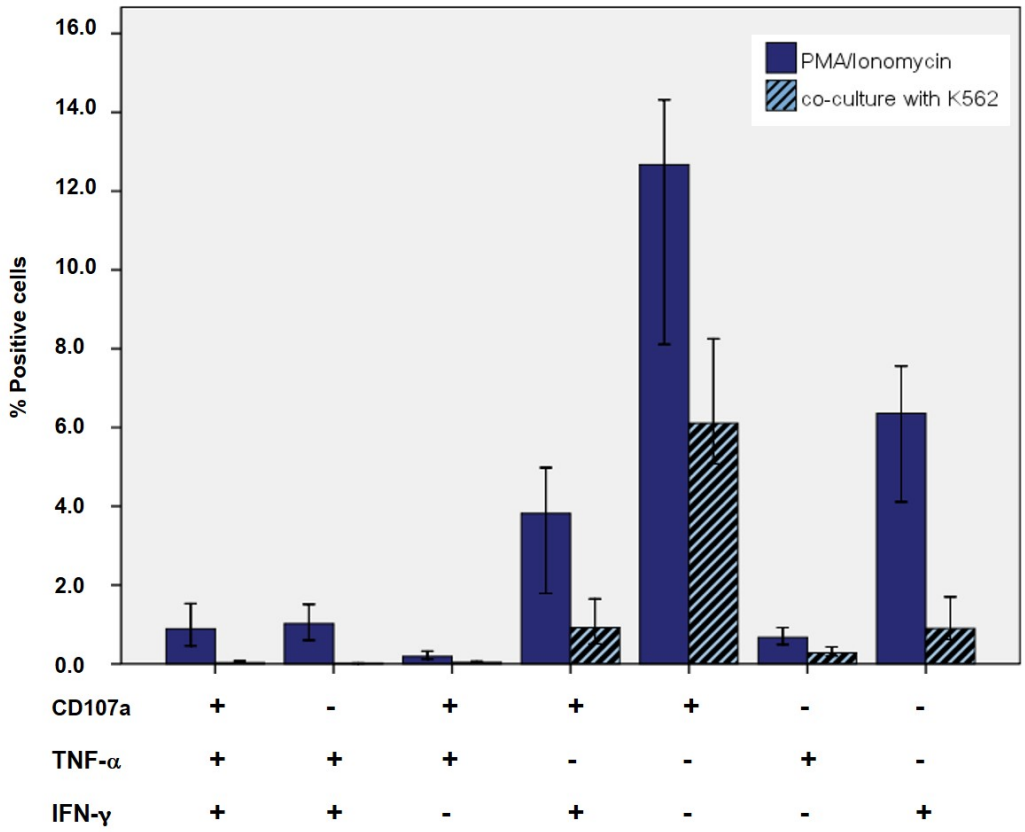

Figure 4 Polyfunctional NK cells in healthy individuals. The functional profiles of NK cells are determined by the possible combination of the expression of CD107a and the production of TNF-α and IFN-γ. The median percentage of NK cell functions in each profile after stimulation with PMA/Ionomycin (filled bar) and K562 (stripe bar) are shown. The x-axis indicates each functional profile of NK cells and the y-axis indicates the percentage of NK cells that expressed markers of functions.

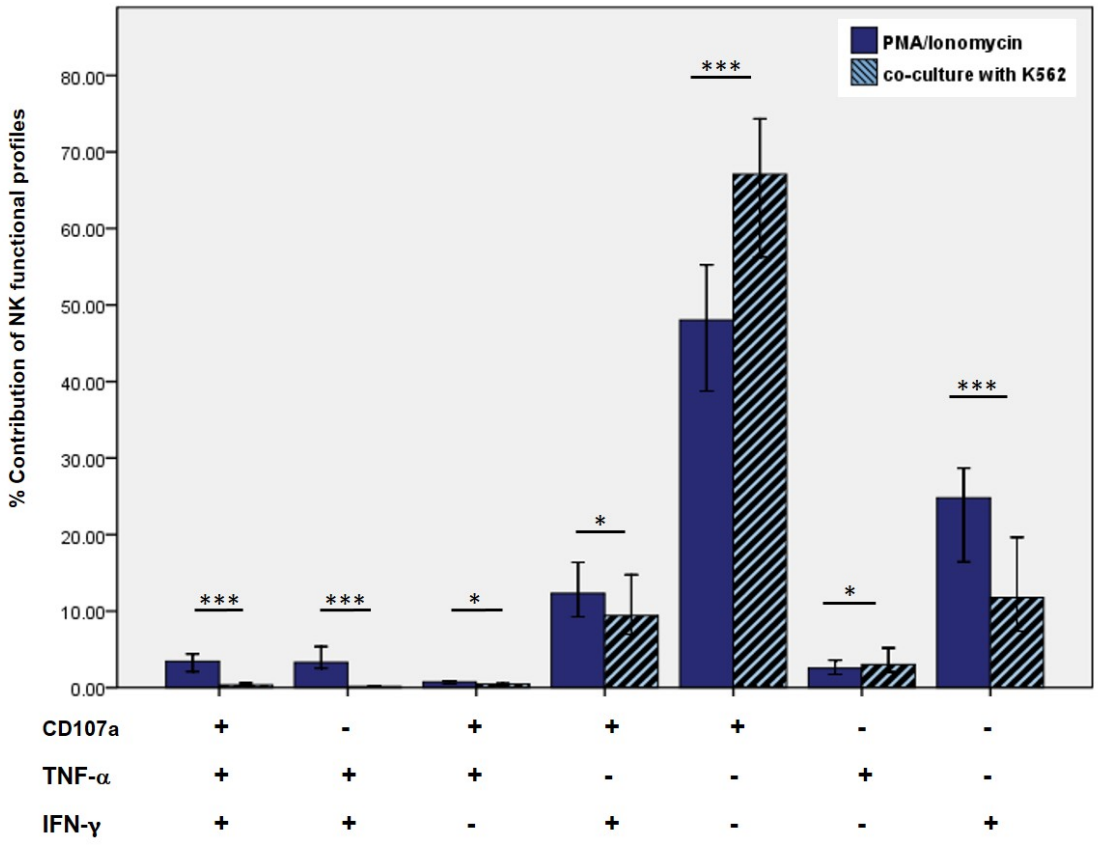

Figure 5 Functional potential of normal NK cells. Seven functional profiles, which are represented in percent contributions, are compared. The percent contribution of each functional subset was calculated using the number of positive NK cells in each functional profile per total functional marker positive NK cells. The bar graph represents the median percent contribution of NK response to stimulations, PMA/Ionomycin stimulation (filled bar) and K562 stimulation (stripe bar). The x-axis indicates each functional profile. The y-axis indicates the percentage of functional NK cells in groups and the error bar of 95 % confidence intervals (CI). Significance between stimuli is shown with an asterisk (*) over the line linking two bars. A dependent student t-test was used to assess the significance of comparisons. Significant levels are shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Discussion

In this present study, we analyzed the capability of NK cell functions in 41 healthy donors. The quality of NK cell effector functions were measured by the expression of CD107a (degranulation), TNF-α, and IFN-γ (cytokine production) after cell stimulation. Multicolor flow cytometry was used, which allowed us to evaluate the 3 effector functions of NK cells simultaneously.

Our study found that the percentage of the majority of NK cell subsets, $CD56^{dim}$ $CD16^{+}$, was similar to previous studies, whereas the percentage of minor subsets, the $CD56^{bright}$ $CD16^{+/-}$ NK cell subset, was lower than previous studies [1,16,17]. However, the similar percentages have been reported in the Thai population by using the same technique [18].

Following NK cell stimulation, we found that activating NK cells predominantly expressed CD107a, followed by IFN-γ and TNF-α, respectively. The number of functional NK cells in response to

PMA/Ionomycin stimulation was higher than that of K562 stimulation. According to NK cell stimulation, PMA/Ionomycin activated NK cells in a surface receptor independent manner, while K562 stimulation required ligands specific to NK cell receptors. In general, the $CD56^{bright}$ NK cell subset was consider to be the major source of cytokines, whereas the $CD56^{dim}$ NK cell subset was regarded as being specialized for cytolytic function [17,19,20]. Therefore, K562 stimulation activated mainly the $CD56^{dim}$ NK cell subset, while PMA/Ionomycin activated both the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets.

The ability of NK cells to perform multiple functions was found in a small fraction of NK cells following stimulation. The major subset was the bifunctional $CD107a^{+}$ and IFN-γ^{+} NK cell subset. The trifunctional NK subset could be found in less than 1 % of the total NK cells. A previous study suggested the correlation between the target cell lysis by NK cells and the expression of CD107a on the surface of NK cells. Therefore, this functional marker of NK cells could be used to discriminate subpopulations of NK cells in response to stimuli [21]. Our data indicated a similar proportion of polyfunctional NK cells in total functionality active cells following PMA/Ionomycin or K562 stimulation. However, the proportion of NK cells which expressed CD107a following K562 stimulation was higher than in that of PMA/Ionomycin stimulation. This phenomenon might be due to the preferential activation of the $CD56^{dim}$ NK cell subset, the highly cytotoxic activity subset, by K562 cells. Additionally, the activity of $CD107a^{+}$ cytokine producing NK cell subsets was not fully characterized. Thus, further study is required for better understanding the role of these polyfunction NK cell subsets in diseases.

Conclusions

In healthy individuals, the NK cells predominantly performed either cytolytic function or cytokine production following stimulation. The polyfunctional NK cells were found in a small fraction of NK cells which highly contributed to cytolytic function and IFN-γ production following stimulation. The trifunctional NK subset could also be found in less than 1 % of the total NK cells.

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