ORIGINAL

Flow Cytometric Evaluation of Surface CD56 Expression on Activated Natural Killer Cells as Functional Marker

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Abstract : Surface CD56 is the most important cell marker for defining NK cells. However, the relationship between the expression of surface CD56 and NK cell activity has not yet been elucidated in detail. Thirteen healthy volunteers were enrolled in the present study. Peripheral blood mononuclear cells (PBMCs) were stimulated with rIL-2 or rIL-12 (1, 10, 100 U/mL) for 18 h at 37℃. After incubation, surface CD56 expression on NK cells was evaluated using a flow cytometric analysis. A colorimetric-based lactate dehydrogenase (LDH) assay was used for experiments on cytotoxicity. IFN-γ mRNA gene expression was quantified by real-time PCR. The expression level of surface CD56 on NK cells, cytotoxicity, and IFN-γ mRNA gene expression were significantly increased by the rIL-2 and rIL-12 stimulations. In addition, a positive correlation was found between surface CD56 expression and cytotoxic activity or IFN-γ mRNA gene expression. We revealed that the quantification of surface CD56 expression was applicable to the evaluation of cytotoxicity and IFN-γ production in activated NK cells. These results suggest that the measurement of surface CD56 expression represent an easy and rapidly reproducible technique to evaluate the activated state of NK cells and monitor NK cell activity in immunotherapy.
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INTRODUCTION

Natural killer (NK) cells were recognized as a separate lymphocyte lineage, with cytotoxicity and cytokine-producing effector functions (1). NK cells comprise approximately 10% of all lymphocytes in human peripheral blood (2), and are important effector cells in the innate immune system because they possess the unique ability to directly lyse malignant-transformed, virus-infected cells without prior sensitization and MHC class restriction (3). NK cell cytotoxicity is mediated by the directed exocytosis of cytolytic granules to release perforins and granzymes, which perforate the target cell plasma cell membrane and trigger apoptosis, respectively (4, 5). Furthermore, NK cells have been shown to play an important role in immune responses by producing interferon gamma (IFN-γ), the production of which by NK cells is known to shape Th1 immune responses (6), activate APCs to further upregulate MHC class I and increase APC cytokine secretion (7), activate the macrophage killing of obligate intracellular pathogens (8), and have anti-proliferative effects on viral- and malignant-transformed cells (9).

Although a large number of surface molecules on NK cells have so far been identified as NK cell markers and receptors, sensitive and truly specific pan-NK-cell markers have not yet been reported. NK cells are currently defined phenotypically by their lack of expression of CD3 and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells (10-12), i.e., $CD3^{-}CD56^{+}$ cells account for most of the NK cell subset. CD56 was previously shown to be increased upon the activation of NK cells (13), and it is one of the adhesion molecules involved in the interaction between NK cells and target cells (14). However, to the best of our knowledge, the relationship between CD56 expression and NK cell activity has not yet been elucidated in detail. Thus, we were interested in the utility of a functional analysis of NK cells. In the present study, we examined the relationship between NK cell-associated immunoreactivity and the expression level of CD56 on human NK cells.

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MATERIALS AND METHODS

Ethics Statement

The study was approved by the Ethics Committee for Human Genome/Gene Research of the University of Tokushima and the Ethics Committee for Kagawa Prefectural University of Health Sciences. Written informed consent was obtained from all volunteers, and all participants signed consent forms approved by the Ethics Committee.

Study participants

Thirteen participants were enrolled from the Kagawa Prefectural University of Health Sciences in this study (7 males and 6 females ; mean age±SD, 23.5±3.7 years). A flow cytometric analysis of surface CD56 expression on NK cells was performed for all participants. Cytotoxicity assay were performed for 10 participants (6 males and 4 females ; mean age±SD, 22.0±1.2 years), while an IFN-γ mRNA gene expression analysis was performed for 10 participants (6 males and 4 females ; mean age±SD, 24.1±4.1 years). Seven participants overlapped between measurement of

cytotoxicity and IFN-γ mRNA gene expression analysis.

PBMC isolation and Cell cultures

Human PBMCs were separated using a gradient centrifugation method with Ficoll-Paque PLUS (GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer's protocol. PBMCs were washed two times with PBS, resuspended in RPMI 1640 medium (Thermo Fisher Scientific, Inc., MA, USA), supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Inc.), and cultured at 37°C in the humidified atmosphere of a 5% CO_2 incubator. A total of two million cells/mL PBMCs were stimulated with rIL-2 or rIL-12 (1, 10, 100 U/mL ; BioLegend, Inc., CA, USA) for 18 h at 37°C, and were then washed two times with PBS before further manipulation.

Flow cytometric analysis of surface CD56 expression on NK cells

In order to detect the expression of surface CD56 on NK cells, PBMCs (1 million cells in 100 μL PBS) were incubated with an appropriate volume of PerCP-conjugated anti-CD3-derived UCHT1 (BioLegend, Inc.) and PE-conjugated anti-CD56PE-derived C5.9 (Exalpha Biologicals, Inc. MA, USA) for 15 min at 4°C. Negative controls were then used under the same conditions of each mouse IgG isotype mAbs. PBMCs were washed two times with PBS, and resuspended in 500 μL of 1 v/v % paraformaldehyde in PBS. PBMCs were analyzed with Cell Lab Quanta SC (Beckman Coulter, Inc., CA, USA) flow cytometer equipped with a 488-nm laser air-cooled configuration. As shown in Fig. 1, lymphocytes were first selected in region 1 (R1) on an electric volume (EV) and side scatter (SS) dot plot (Fig. 1A). Among the cells selected in gate R1, NK cells were selected in a second region (R2) as $CD3^-CD56^+$ cells on an FL-2 (CD56-PE)/FL-3 (CD3-PerCP) dot plot (Fig. 1B). NK cells were defined as $CD3^-CD56^+$ cells, and the expression of CD56 on these cells was quantified by geometric mean fluorescence intensity (GMFI) (Fig. 1C). For each sample analyzed, a minimum of 20,000 events was acquired. All measurements were performed under the same compensation settings.

Cytotoxicity assay

Cytotoxicity assay was performed using stimulated PBMCs as effector cells. PBMCs were suspended at 1×10^6 cells/mL in RPMI 1640 medium without phenol red. Raji cell line (JCRB Cell Bank, Osaka, Japan) was used as the target cell. Raji cells were suspended at 5×10^4 cells/mL, and co-cultured with effector cells (50 μL/well each) for 4h on a 96-well microplate at 37°C with 5% CO_2. The effector to target (E : T) cell ratio was 20 : 1. A colorimetric-based lactate dehydrogenase (LDH) assay (Cytotoxicity Detection Kit^{PLUS} ; F. Hoffmann-La Roche, Ltd., Basel, Switzerland) was used and cytotoxic activity was calculated according to the manufacturer's instructions. Cell-mediated cytolysis were converted to percent specific lysis (% SL) using the following formula : % SL=[(experimental LDH release-spontaneous LDH release)/(maximum LDH release-spontaneous LDH release)]×100.

IFN-γ mRNA gene expression analysis

After stimulation with rIL-2 or rIL-12, NK cells were separated from PBMCs using the EasySep Human NK Cell Enrichment Kit (STEMCELL Technologies, British Columbia, Canada) to determine IFN-γ mRNA expression in NK cells. Total RNA was extracted using the QIAamp RNA Blood Mini Kit (Qiagen) from NK cells according to the manufacturer's protocol. cDNAs were obtained from mRNA by reverse transcription (15 min at 42°C, 2 min at 95°C) in a final volume of 20 μL, containing 1.0 ng of total RNA, 2.0 μL of 10×PCR buffer, 5.0 mM $MgCl_2$, 0.5 mM dNTP, 2.5 μM Random hexamer primer, 1.0 U/μL RNase inhibitor, and 2.5 U/μL MuLV Reverse Transcriptase (Thermo Fisher Scientific, Inc.).

The relative quantification of IFN-γ mRNA gene expression was analyzed in hot-start qPCR using LightCycler FastStart DNA Master SYBR Green I (Roche). In order to internally standardize gene expression levels, we used a GAPDH housekeeping gene. Amplifications were performed with LightCycler 2.0 (Roche) in a final volume 20 μL, containing 5.0 μL of cDNA, 2.0 μL of 10×Master Mix, 3.0 mM $MgCl_2$, and 1.0 μM of each primer. PCR amplification conditions were an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 62 to 58°C (Δ0.5°C/cycle) for 10 sec, and extension at 72°C for 16 sec, followed by a quick chill to 4°C. GAPDH-specific primers were as follows : forward 5'-GCACCGTCAAGGCTGAGAAC-3' ; reverse 5'-TGGTGAAGACGCCAGTGGA-3', and IFN-γ specific primers were as follows : forward, 5'-AAAAATAATGCAGAGCCAAATTG-3' ; reverse, 5'-TAGCTGCTGGCGACAGTTCA-3'. Each reaction was performed in triplicate, and the relative expression of IFN-γ mRNA was obtained using the ΔCT method. Lower ΔCT values indicated higher mRNA levels.

Statistical analysis

Results were expressed as the mean±standard deviation. In order to compare the treatment group to a single control group, we assessed significance with the Dunnett's test, which is a multiple comparison procedure. A Spearman's rank correlation was applied to determine the relationship between different study parameters. P values below 0.05 were considered significant. IBM SPSS Statistics 22.0 software was used to perform statistical analyses.

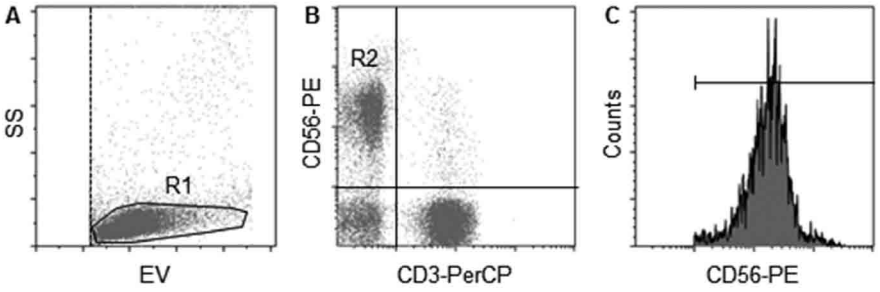

Fig. 1. Flow cytometric analysis of surface CD56 expression levels on NK cells. These Figures are from a single representative subject. Lymphocytes were selected in region 1 (R1) on an electric volume (EV) and side scatter (SS) dot plot (A). NK cells (R2) were defined as $CD3^-CD56^+$ cells (B), and surface CD56 expression on these cells was quantified by geometric mean fluorescence intensity (GMFI) (C).

RESULTS

Expression levels of surface CD56 on NK cells after an in vitro stimulation

Flow cytometric analysis was performed for quantification of surface CD56 expression on NK cells. GMFI indicated fluorescence intensity of a population of cells. The expression levels of surface CD56 on NK cells following a stimulation with rIL-2 or rIL-12 were shown in Fig. 2. Surface CD56 expression was significantly increased by rIL-2 (Fig. 2A) and rIL-12 (Fig. 2B).

Cytotoxic activity after an in vitro stimulation and relationship between cytotoxic activity and expression of surface CD56

The cytotoxicity assay was performed using stimulated PBMCs as effector cells. The cytotoxic activity was determined with a LDH assay. The cytotoxic activity of PBMCs following a stimulation with rIL-2 or rIL-12 were shown in Fig. 3. The cytotoxic activity was significantly enhanced following the stimulations with rIL-2 (Fig. 3A) and rIL-12 (Fig. 3B).

Fig. 3C and 3D show the relationship between cytotoxic activity and the expression of surface CD56 on NK cells following a stimulation with rIL-2 or rIL-12. The expression of surface CD56 correlated with cytotoxic activity (Fig. 3C : stimulation with rIL-2 ; $r=0.667$, $p<0.001$, Fig. 3D : stimulation with rIL-12 ; $r=0.832$, $p<0.001$).

Expression levels of IFN-γ mRNA after an in vitro stimulation and relationship between IFN-γ mRNA expression levels and expression of surface CD56

IFN-γ mRNA expression was quantified by real-time PCR. In order to internally standardize gene expression levels, we used a GAPDH as housekeeping gene. The amount of IFN-γ mRNA expressed was depicted as ΔCT values. Lower ΔCT values indicated higher mRNA levels. Fig. 4A and 4B show IFN-γ mRNA expression levels following a stimulation with rIL-2 or rIL-12. As shown in Fig. 4, the ΔCT values of IFN-γ mRNA was significantly decreased by the stimulations with rIL-2 (Fig. 4A) and rIL-12 (Fig. 4B), i.e., IFN-γ mRNA expression levels were significantly increased after the *in vitro* stimulation. A negative correlation was also observed between CD56 GMFI and IFN-γ mRNA ΔCT values (Fig. 4C : stimulation by IL-2 ; $r=-0.839$, $p<0.001$, Fig. 4D : stimulation by IL-12 ; $r=-0.813$, $p<0.001$). In other words, CD56 expression levels and IFN-γ mRNA expression levels were positively correlated.

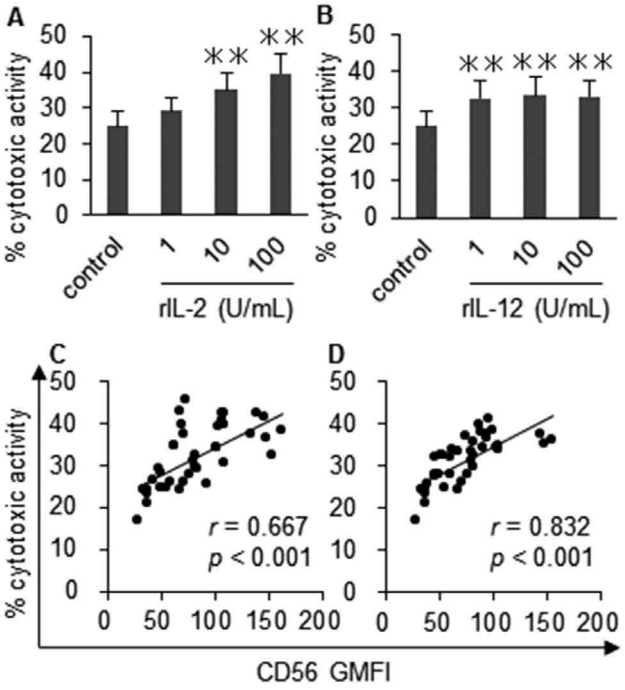

Fig. 3. Cytotoxic activity and its relationship to surface CD56 expression on NK cells. Cytotoxic activity was determined using PBMCs as effector cells. Cytotoxic activity was analyzed after a stimulation with rIL-2 (A) or rIL-12 (B). Scatter diagrams represent the relationship between CD56 GMFI and cytotoxic activity after a stimulation with rIL-2 (C) or rIL-12 (D). Results are expressed as the mean±standard deviation of 10 subjects. Significant differences were assessed to compare the treatment group to a single control group (**$p<0.01$; Dunnett's test).

DISCUSSION

NK cells are large granular lymphocytes that play a role in the cellular recognition and killing of virus-infected and tumor cells, and their activation is important in the immune system *in vivo.* Previous clinical studies reported the close relationship between NK cell activity and various diseases. Konjevic *et al.* showed that breast cancer patients in all clinical stages of the disease had significantly decreased NK cell activity, and this decrease was the

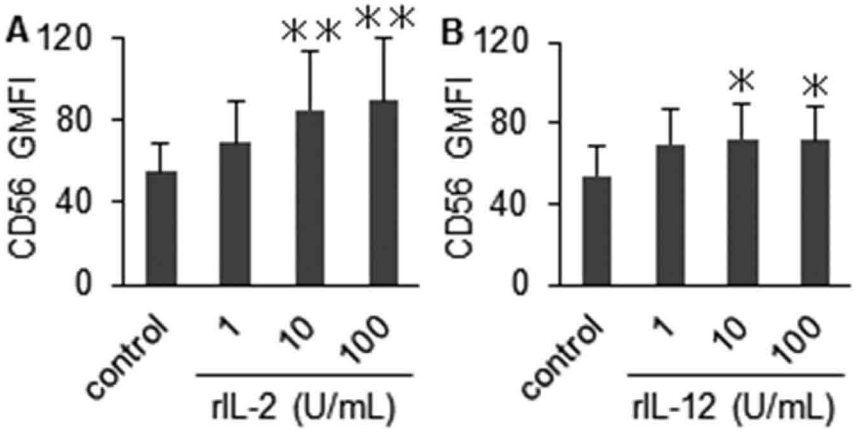

Fig. 2. Expression levels of surface CD56 on NK cells following a stimulation with rIL-2 or rIL-12. PBMCs were stimulated with rIL-2 or rIL-12. CD56 GMFI was analyzed after the stimulation with rIL-2 (A) or rIL-12 (B), respectively. Results are expressed as the mean±standard deviation of 10 subjects. Significant differences were assessed to compare the treatment group to a single control group (*$p<0.05$, **$p<0.01$; Dunnett's test).

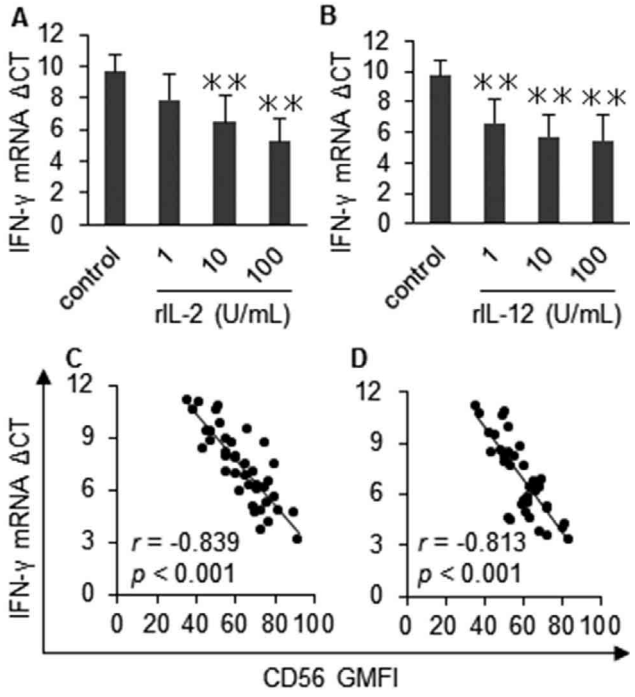

Fig. 4. Expression levels of IFN-γ mRNA and its relationship to surface CD56 expression on NK cells. PBMCs were stimulated with rIL-2 or rIL-12. IFN-γ mRNA ΔCT values were analyzed from separated NK cells after a stimulation with rIL-2 (A) or rIL-12 (B). Scatter diagrams represent the relationship between IFN-γ mRNA ΔCT values and CD56 GMFI after a stimulation with rIL-2 (C) or rIL-12 (D). Lower ΔCT values indicated higher mRNA levels. Results are expressed as the mean± standard deviation of 10 subjects. Significant differences were assessed to compare the treatment group to a single control group (**$p < 0.01$; Dunnett's test).

greatest in the advanced stage of the disease (15). Furthermore, they revealed that the clinical stage of the disease also contributed to the degree of NK cell dysfunction in non-Hodgkin's lymphoma, and NK cell activity was persistently decreased irrespective of the histological type or clinical stage in Hodgkin's lymphoma (16). Furthermore, previous studies showed that the number of circulating NK cells was reduced concomitant with a decrease in NK cell cytotoxicity in several cases of autoimmune diseases (17-21). Therefore, a detailed functional analysis of NK cells may lead to the development of new therapies and diagnoses for various diseases. Functional analyses of NK cell activation are currently evaluated based on cytotoxicity and cytokine production.

NK cells express many different molecules on their cell surface membrane. Of these, CD56 has been identified as the surface marker that best characterize NK cells. CD56 is an important surface marker for distinguishing NK cells (22), and has been reported to increase following the activation of NK cells (13). However, to the best of our knowledge, the relationship between CD56 expression and NK cell activity has not yet been elucidated in detail. Thus, we were interested in the utility of a functional analysis on NK cells. In the present study, we attempted to establish a human NK cell activity evaluation method based on a quantitative flow cytometric analysis of CD56 expression levels in NK cells.

In this study, we investigated changes in CD56 expression levels, cytotoxic activity, and IFN-γ production with NK cell activity as well as the relationships between cell membrane CD56 expression and cytotoxicity or IFN-γ production. The activation of NK cells was stimulated by rIL-2 and rIL-12. IL-2 is known to promote NK cell proliferation, cytotoxicity, and cytokine secretion. In humans, the lymph nodes, in which $CD4^+$ T cells and NK cells interact, may be the location at which T cell-derived IL-2 boosts NK cells (23). In addition, the secretion of IL-12 by plasmacytoid DCs was shown to increase NK cell proliferation, cytotoxicity, and IFN-γ production (24). In the present study, we demonstrated that the expression level of CD56 on NK cells, cytotoxic activity, and IFN-γ production were significantly increased by the rIL-2 and rIL-12 stimulations and in a concentration-dependent manner by the rIL-2 stimulation. Surface CD56 expression was also significantly increased in case of using isolated NK cells from PBMCs (data not shown). In addition, there was no significant change of proportion between $CD56^{dim}$ and $CD56^{bright}$ subsets by a stimulation, and we observed upregulation of CD56 expression in the overall NK cells (data not shown). Furthermore, a positive correlation was found between CD56 expression levels and cytotoxic activity or IFN-γ mRNA expression. These results indicated that the immune functions of activated NK cells were evaluable based on the expression level of CD56 on NK cells. Measurement of cytotoxic activity has widely been used to evaluate the immune functions of NK cells, but this assay is complex. In this study, we measured IFN-γ mRNA expression to evaluate capacity of IFN-γ production because IFN-γ products in cell culture supernatant indicated accumulated amount during stimulation.

A flow cytometric analysis for the quantification of CD56 expression can be performed easily and quickly. Recently, C-type lectin-like cell receptors (CD69), the killer cell lectin-like receptor G1 (KLRG1), and the lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) have been reported as markers of activated NK cells. However, these cell surface markers are not expressed on resting NK cells (25). In contrast, CD56 is expressed not only on activated NK cells, but also on resting NK cells. Comparison between surface CD56 expression and CD107a expression on activated NK cells have still to be investigated. We suggest that surface CD56 expression on NK cells is useful for evaluating NK cell functions.

CONCLUSION

The quantification of CD56 expression levels is applicable to the evaluation of cytotoxic activity and IFN-γ production in activated NK cells. The measurement of CD56 expression represents an easy and rapidly reproducible technique to evaluate the activated state of NK cells and monitor NK cell activity in immunotherapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this manuscript.

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