Chapter 8

A New Method to Determine Natural Killer Cell Activity Without Target Cells

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http://dx.doi.org/10.5772/intechopen.71912

Abstract

Natural killer (NK) cell activity is a conventional parameter used to determine the performance lytic activity against tumor as well as virus-infected cells in innate immunity. However, use of this parameter has several problems related to bioassay measurements. To measure NK cell activity, target cells and cell culture equipment are required and adequate pre-culture of target cells is needed to maintain constant sensitivity for NK cells. NK cell-activating receptors play an important role in the recognition of targets, which transduce the signals necessary for cellular machinery to induce target injury and cytokine production. We statistically examined the parameters related to the NK cell activity of human peripheral blood mononuclear cells (PBMCs) by multiple regression analysis, and obtained a formula with NK cell % and RNA levels of two genes in isolated NK cells. The score calculated using this formula with the three measured values showed significant correlation with NK cell activity. This prediction score, named the non-incubating natural killer (NINK) score, which is independent of target cells, is not affected by inappropriate preparation of those targets, and allows us to accurately compare the performance of NK cell activity among specimens.

Keywords: NK cell activity, activating receptor, NKp46, IFN-γ

1. Introduction

The characteristics of antitumor immunity in the body are of interest not only with respect to healthy individuals but also in relation to patients diagnosed with certain kinds of tumor diseases. With the former, information can be utilized by individuals to assess

their lifestyle so as to prevent the occurrence of tumor diseases. If smokers were aware of decreased levels of their antitumor immune functions, they might be more motivated to quit smoking or to have medical checks more often. As these considerations are invaluable in the area of preventive medicine, and have the potential to decrease the national cost of medical expenses, the development of appropriate and easy-to-use devices for measuring the status of tumor immunity would be desirable. In clinical medicine, doctors also hope to determine alterations in immunological status as well as tumor size in the body of patients following cancer therapy. The immunological information obtained following the treatment might contribute to an adequate determination of the therapeutic efficacy by doctors. Natural killer (NK) cell activity testing is one effective approach to determine the status of antitumor immunity in the body, which is reflected from the crosstalk between cancer cells and immune cells and the nature of those immune cells. NK cell activity is a conventional index which represents the ability of cell samples to injure NK cell-sensitive target cells, and we utilized the method of NK cell activity to evaluate the natural cytotoxic activity of peripheral blood mononuclear cells (PBMCs) obtained from patients and an NK cell line [1–5]. A recent 11-year follow-up study from 1986 to 1990 involving 3652 Japanese residents of the general population clearly demonstrated the importance of determining and evaluating NK cell activity. In that report, individuals with low NK cell activity actually showed higher cumulative incidence rates of cancer compared to people with high or medium NK cell activity, regardless of gender [6]. In particular, women showed more differences in the incidence rate of cancer, which represented about a twofold difference between groups with low and high or medium levels of NK cell activity. Thus, NK cell activity is a good index in evaluating the status of antitumor immunity. In fact, our previous studies measuring NK cell activity demonstrated that indoor air conditions have a potential to interfere with NK cell function [7–10]. However, NK cell activity testing possesses several difficulties, which concerns researchers and doctors when considering the potential use of NK cell activity as an index in basic and clinical studies in medical science. In the next section, these issues will be examined.

2. The conventional method to examine NK cell activity

Simplicity is the reason why the conventional method of determining NK cell activity has been a standard till date. With this method, NK cell-sensitive targets such as K562 cells are prepared and cell specimens are incubated with the target cells for 4 h at 37°C in a CO_2 incubator. The original "^{51}Chromium release assay (CRA)" method was developed during the 1960s [11, 12]. With that original method, researchers have to label target cells with the ^{51}Cr radioisotope in an effort to determine the amount of radioisotope released from targets lysed by NK cells in specimens, which reflects the amount of killed targets or, in other words, the NK cell activity. Although to date, commercial services have been examining NK cell activity by employing the ^{51}Cr release assay, researchers have the option of using flow cytometry with fluorescence dyes *in lieu* of radioisotopes to measure NK cell activity. This approach can distinguish targets from effectors by labeling targets with a fluorescence dye such as carboxyfluorescein diacetate succinimidyl ester (CFSE) and DiO, which has similar excitation

and emission properties as fluorescein isothiocyanate (FITC). Then, since dead targets can be distinguished from viable cells by staining with propidium iodide (PI), the percentage of PI^+ targets in the total can be measured using flow cytometry in a radioisotope-free manner. Actually, we have measured NK cell activity in this manner and understand the usability of this conventional method [1, 2], although we became familiar with several kinds of difficulties. In order to assay for NK cell activity, a researcher needs to decide the date for the assay and consequently adjust target cells to an adequate condition by pre-culturing for a fixed number of days at a fixed cell density, to ensure that the target cells operate with consistent sensitivity on the assay day. Therefore, although a researcher can assay for NK cell activity of a scheduled specimen on one day under the same conditions performed on the previous day, it may be difficult to maintain adequate or appropriate conditions in the assay for use of an unscheduled specimen since the pre-culture of target cells has not been accomplished on the day. We demonstrated that differences in pre-culture conditions of target cells leads to alterations in the results obtained from the assay for NK cell activity (**Figure 1**). The specimen comprising PBMCs was prepared from human peripheral blood and used as effectors equally for the assays, while K562 cells were prepared as targets by pre-culturing at two different cell

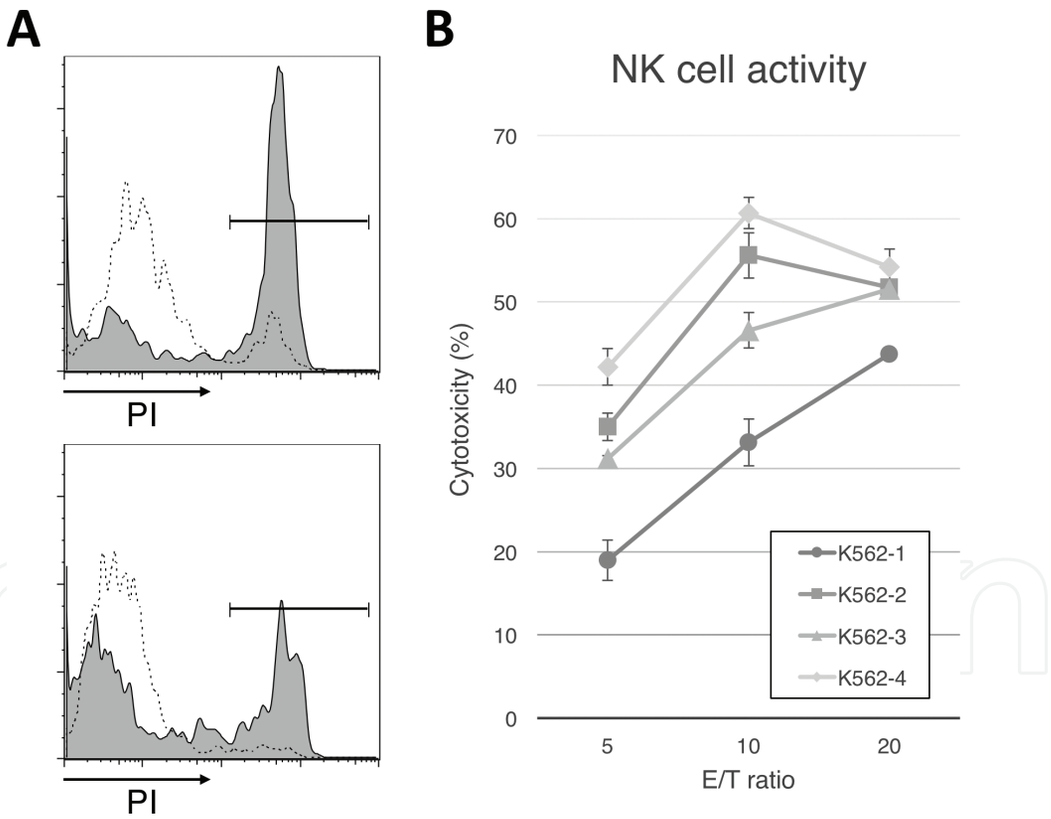

Figure 1. Variation in the results of assaying for NK activity due to differences in the pre-culture conditions of target cells. K562 cells were pre-cultured under four different conditions using two different cell densities prepared separately by two individuals. A shows representative histograms of K562 cells obtained by flow cytometry with a high percentage of PI^+ dead cells (upper) and low percentage of cells (lower) at an E/T ratio of 10 following incubation with human PBMCs for 4 h. K562 cells were distinguished from PBMCs by positive DiO staining and gated to show the histograms. The bar represents the region defined and measured as the population of DiO^+PI^+ cells. Dashed lines represent targets alone. A summarized graph with mean and SD from the three wells is shown in B. It is clear that the cytotoxicity obtained from the assay with K562-1 shows only half the degree of cytotoxicity obtained from the assay with K562-4.

densities prepared separately by two individuals. Following pre-culturing, all targets were stained with DiO and incubated with PBMCs in a 96-well U-bottom culture plate for 4 h. Following incubation, cells were stained with PI before being analyzed by flow cytometry, where the percentage of DiO^+PI^+ cells in the total number of DiO^+ cells was measured as lysed target cells. Finally, the percentage of natural cytotoxicity was calculated as follows:

$$\text{NK cell activity (\%)} = \left([\text{percentage of lysed cells}] - [\text{percentage of spontaneously dead cells}]\right)/\left(100 - [\text{percentage of spontaneously dead cells}]\right) \times 100 \quad (1)$$

In this formula, the percentage of spontaneously dead cells represented the percentage of dead cells in target cells harvested from wells without effector cells. In that experiment, the values of NK cell activity clearly differed among the four kinds of pre-cultured K562 cells and showed a maximum twofold difference at an E/T ratio of 10. These results indicate that the values of NK cell activity vary between assays, a phenomenon which is unavoidable when using target cells, that is, bioassay. If researchers were not bound to use target cells for the determination of NK cell activity without targets, the results obtained from the assays might be more stable, and requirements such as a CO_2 incubator and a clean bench for cell culture would be unnecessary. Therefore, we attempted to develop a new method to determine NK cell activity without the use of cell culture.

3. The importance of NK cell-activating receptors in NK cell cytotoxicity

Although both NK and $CD8^+$ T cells have the ability to injure target cells, these cells also possess many different characteristics, one of which is the machinery required for the recognition of targets followed by signal transduction linked to cell injury. The diversity of antigen specificity in T cells is dependent on the T cell receptor (TCR) complex on the cell surface, and accounts for the ability of $CD8^+$ T cells to recognize and kill any kind of target cell by the strong interaction between the TCR and MHC antigen peptide complex. However, as naïve T cells are not ready to exert target injury and antigen specificity differs among cells, with only a small amount being present within each clone, $CD8^+$ T cells need to be activated before injuring targets. In contrast, NK cells are capable of killing targets with no activation required and are equipped with various kinds of receptors to recognize targets, referred to as NK cell-activating receptors (KARs) [13–16]. NKG2D is the most-studied of these receptors and belongs to the NKG2 family of proteins which are characterized by the presence of a lectin-like domain. NKG2D binds to MHC class I polypeptide-related sequence A and B (MICA/B) and UL16-binding protein (ULBP) [17–22], which are often expressed in tumor cells [23, 24]. Natural cytotoxicity receptors (NCRs) also play a role in killing various kinds of tumors, and NKp30, NKp44, NKp46 and NKp80 are members of the NCR family of proteins [14]. Moreover, the signaling lymphocyte activating molecule (SLAM) family are another group of players involved in the recognition of targets by NK cells, and 2B4 (CD244), a representative member of the SLAM family, recognizes CD48 and leads to cytotoxicity [25–29]. It is thought that the

http://dx.doi.org/10.5772/intechopen.71912

variety of activating receptors on a single cell impart NK cells with the ability to exert cytotoxicity against various target cells without clonal selection and expansion as with T lymphocytes. Those activating receptors share the same mechanism of signal transduction, by which a microtubule organizing center (MTOC) is induced to polarize cytotoxic granules, including perforin and granzymes, near the plasma membrane, and those intragranular molecules are subsequently released against targets *via* degranulation (**Figure 2**). The ligation of those activating receptors allow Src family kinases (SFK) to trigger the pathways from phosphoinositide -3 kinase (PI3K) to extracellular signal-regulated kinase (ERK) and from phospholipase C (PLC) γ to c-jun N-terminal kinase (JNK) to facilitate polarization [30–32]. Those findings led us to surmise that some alteration in cell surface expression levels of activating receptors on NK cells might influence lytic activity against targets. We previously investigated the toxicological effects of asbestos on NK cell function, and demonstrated that asbestos exposure caused impaired cytotoxicity of NK cells with altered expression of several activating receptors [1, 2, 4, 5]. Continuous exposure of the human NK cell line YT-A1 to asbestos resulted in decreased levels of cell surface NKG2D and 2B4, as well as impaired cytotoxicity against K562 cells. Furthermore, it was confirmed that the degranulation induced by stimulation with antibodies to NKG2D or

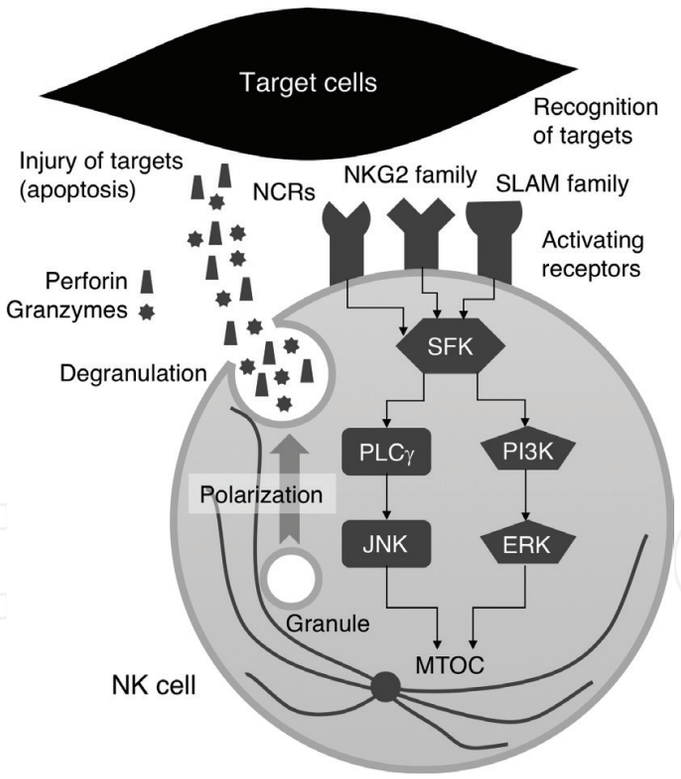

Figure 2. The summarized machinery of target cell injury caused by target recognition with activating receptors. Recognition of target cells with various activating receptors induces Src family kinases (SFK) to trigger the two pathways from phosphoinositide-3 kinase (PI3K) to extracellular signal-regulated kinase (ERK) and from phospholipase C (PLC) γ to c-jun N-terminal kinase (JNK). Both of these pathways induce polarization of cytotoxic granules *via* a microtubule organizing center (MTOC), whereby granules move to a region near the plasma membrane. Finally, the fusion between plasma membrane and granular membrane occurs to induce degranulation, and perforin and granzymes are released from those granules to induce death of the target cells by apoptosis.

2B4 was low in those asbestos-exposed cells. Moreover, we examined the characteristics of human primary NK cells in PBMCs cultured with asbestos and found a decrease in cell surface NKp46 in patients with malignant mesothelioma, a tumor disease caused by inhalation of asbestos, and also showed impaired natural cytotoxicity. Less information is known about the natural ligands of NKp46. However, a previous investigation demonstrated that cell surface expression levels of NKp46 were correlated with the natural cytotoxicity of K562 and that reverse antibody-dependent cell-mediated cytotoxicity (ADCC) of P815 was correlated with antibodies to NKp46 [33]. Additionally, our previous study demonstrated that NK cells in healthy individuals with high natural cytotoxicity showed high expression of NKG2D, NKp46 and phosphorylation of ERK following stimulation *via* those receptors, whereas NK cells in individuals with low natural cytotoxicity showed the converse [2]. These results led us to surmise that determination of the gene expression level of activating receptors might be one important parameter in estimating the natural cytotoxicity of effector cells such as PBMCs *in lieu* of employing methods involving incubation with NK-sensitive target cells.

4. The roles executed by NK cells with molecules in cytotoxic granules, the cell surface ligand and secreted proteins

As mentioned above, the cell surface expression of activating receptors on NK cells is a key event which defines the performance of those cells. What precisely occurs in NK cells following stimulation with KARs? NK cells execute two different events following recognition of target cells with activating receptors (**Figure 3**). The first event exerts natural cytotoxicity against the targets by releasing perforin and granzymes in cytotoxic granules into the space of the immune synapse between NK and target cells. Perforin is thought to function in generating a pore in the plasma membrane as complement proteins, and then granzymes enter through the pore and mediate apoptosis by deploying their serine protease activity [34]. Alternatively, NK cells also express cell surface FasL, which can also induce apoptosis through Fas receptors on the target cells [35]. Moreover, TNF-related apoptosis-inducing ligand (TRAIL) is also produced by NK cells and induces apoptosis of target cells like FasL [36–38]. This natural cytotoxicity itself highlights the importance of early removal of abnormal cells, which transiently appear in the body, and directly contributes to preventing the development of tumor diseases. However, it also has another role linked to antigen-specific cytotoxicity by cytotoxic T lymphocytes (CTLs) in acquired immunity. CTLs have to be primed by dendritic cells (DCs) with a complex comprising antigen peptide and MHC class I molecule before they can become effective cytotoxic cells from naïve cells. In this priming, extracellular antigen is exceptionally presented on MHC class I for CTLs by a particular subset of DCs, in a process referred to as "cross-presentation" [39–41]. These type of DCs endocytose dead target cells, digest their antigens and express a complex of MHC class I and antigen peptide on the cell surface [42], and the dead cells can be provided from early injury of target cells by NK cells to DCs [43]. That is why NK cells are linked to acquired

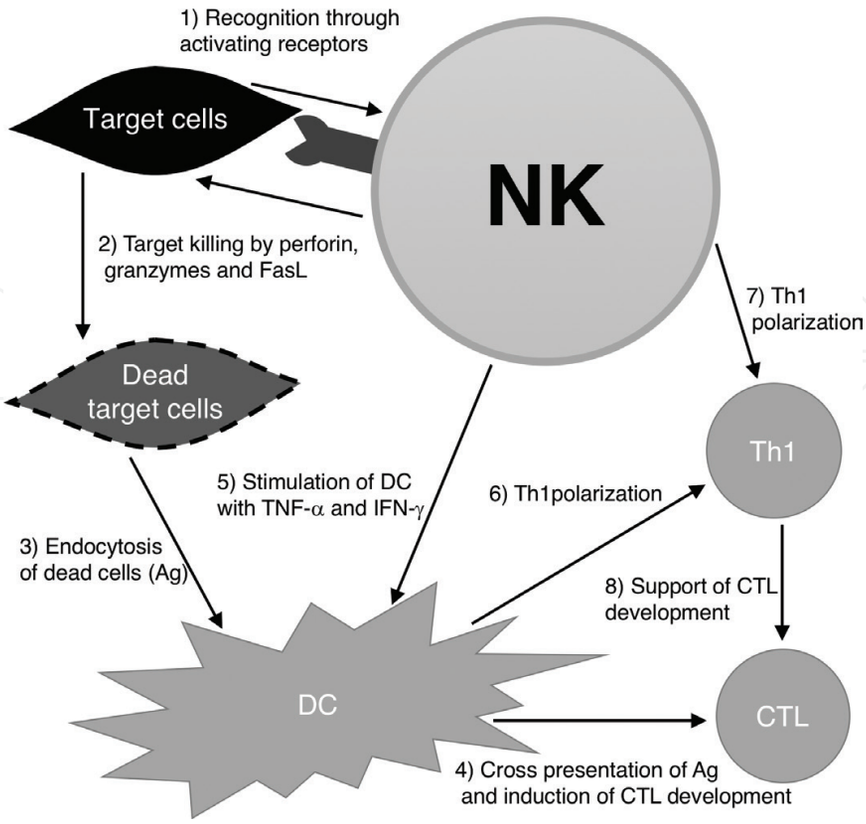

Figure 3. Summarized illustration of the roles executed by NK cells following the recognition of targets with activating receptors and subsequent responses in acquired immunity. (1) NK cells recognize target cells with activating receptors including NKG2D and NKp46, which trigger the machinery for target cell injury. (2) NK cells exert the action of killing targets by perforin/granzymes, FasL or TRAIL. (3) Dead target cells are endocytosed as antigen (Ag) by DCs. (4) The particular subset of DCs which have endocytosed lysed target cells play a role in "cross-presentation" to stimulate naïve $CD8^+$ T lymphocytes to develop into mature CTLs. (5) The stimulated NK cells also produce cytokines including TNF-α and IFN-γ, (6)–(8) which stimulate DCs to produce IL-12 and other proinflammatory cytokines, thereby promoting Th1 cell polarization and CTL development.

immunity as well as function in innate immunity by themselves. Secondly, the production of cytokines including TNF-α and IFN-γ by NK cells after recognition of targets also plays an important role in DC maturation [43, 44]. Those cytokines stimulate DCs to produce IL-12 and other proinflammatory cytokines, which promote Th1 cell polarization and CTL development with specificity against target cells. Additionally, IFN-γ produced by NK cells is able to effect Th1 polarization directly. Those findings relating to the production of cytokines by NK cells, in particular, as IFN-γ is a key cytokine which is produced by NK cells and supports tumor immunity, leads us to hypothesize that the production of IFN-γ by NK cells in an individual might be utilized alone to estimate the performance of natural cytotoxicity in those cells. However, it is known that NK cells can be divided into two populations comprising $CD56^{bright}$ and $CD56^{dim}$ cells, which show different natural cytotoxicity and production of IFN-γ. $CD56^{bright}$ NK cells have high production of IFN-γ and low natural cytotoxicity, whereas $CD56^{dim}$ NK cells have low production of IFN-γ and high natural cytotoxicity [45, 46]. Those findings demonstrate that measurement of IFN-γ production by NK

cells is insufficient to estimate the natural cytotoxicity of those cells and that determination of multiple parameters related to NK cells is necessary in order to effectively evaluate the performance of natural cytotoxicity in an indirect manner. All of this information indicates that the performance of NK cells is reflected by the strength of stimulation through cell surface activating receptors as well as the subsequent production of functional molecules as described above.

5. The development of a new index: non-incubating natural killer (NINK) score

The findings concerning NK cells described above led our research group to surmise that calculation of a prediction score based on several factors that play a role in NK cells might be utilized as an effective index to determine the performance of NK cell activity of PBMCs in an individual without the need to prepare target cells. Therefore, we statistically analyzed factors that may correlate with the NK cell activity of human PBMCs using multiple regression analysis with linear regression model, and in doing so attempted to arrive at a formula to calculate the prediction score of NK cell activity (**Figure 4**) (manuscript of an original article under preparation). In that analysis, the following parameters were used as independent variables for NK cell activity: the percentage of $CD3^{-}CD56^{+}$NK cells (NK%) in PBMCs and mRNA levels of NKp46, granzyme B, FasL, TNF-α and IFN-γ relative to GAPDH mRNA levels in isolated NK cells, which were measured by flow cytometry and real-time PCR, respectively.

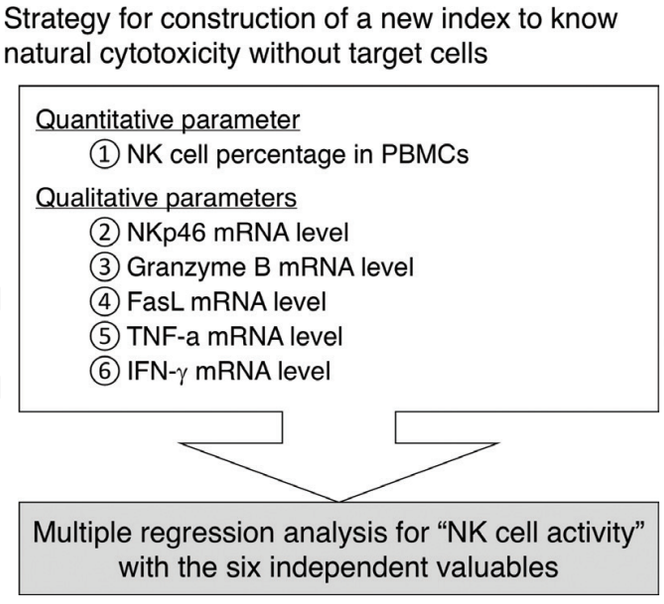

Figure 4. Scheme of strategy for construction of a new index to determine natural cytotoxicity without the use of target cells. The upper box shows six candidate parameters which we selected to explore the new index in an effort to determine natural cytotoxicity. The percentage of NK cells in PBMCs represents the amount of NK cells as a quantitative parameter, while mRNA expression levels of NKp46, granzyme B, FasL, TNF-α and IFN-γ are thought to function or interfere with the strength of lytic activity of NK cells as qualitative parameters. We analyzed the relationship of those parameters with respect to NK cell activity using multiple regression analysis.

The value of the mRNA level, being ΔCq obtained from real-time PCR, was log-transformed (base-10) and used for the multiple regression analysis. The NK% and other parameters were examined as quantitative and qualitative parameters, respectively, and related to the performance of NK cell activity, the reason why those parameters were chosen for that analysis. The conventional index of NK cell activity of PBMCs was assayed using the K562 cell line as the target cells. The results of the multiple regression analysis showed a significant correlation between NK cell activity and NK%, NKp46 mRNA and IFN-γ mRNA, and the prediction formula obtained from the statistical analysis comprises the aforementioned three correlation factors as shown below.

$$\begin{aligned}\text{NINK score} = {} & a + b[\text{NK\%}] + c[\text{NKp46 mRNA in NK cells}] \\ & + d[\text{IFN-gmRNA in NK cells}]\end{aligned} \tag{2}$$

The score calculated using the prediction formula with values of each parameter derived from each individual showed a better Pearson's correlation coefficient with NK cell activity than using either NK%, NKp46 mRNA or IFN-γ mRNA levels alone. These results indicate that this prediction score, named the non-incubating natural killer (NINK) score, can reflect the performance of natural cytotoxicity without the use of target cells to measure NK cell activity (patent pending). Finally, we confirmed the feasibility of the NINK score using another group of individuals. In this experiment, blood in collection tubes was stored overnight in a

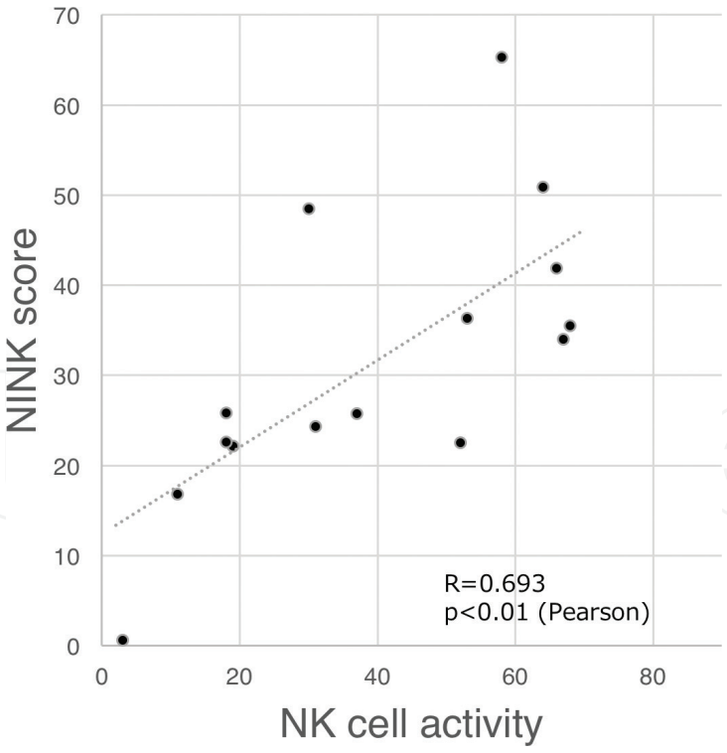

Figure 5. The positive correlation of the NINK score with NK cell activity. The NINK score was calculated using the prediction formula comprising NK% in PBMCs and mRNA levels of NKp46 and IFN-γ in isolated NK cells derived from PBMCs from each individual, and then examined for a correlation with NK cell activity using Pearson's correlation test. The graph shows a significantly positive correlation of the NINK score with NK cell activity. The correlation coefficient and statistical significance of the p value are shown in the graph.

container box at 22°C prior to executing the assays outlined below, since the actual procedures involving PBMC preparation, NK% measurement and isolation of $CD56^+$ NK cells, followed by subsequent measurement of mRNA levels, may need to be performed on the following day after the blood is collected at a distant clinic and then transported to the institute where the subsequent procedures are performed. The results of that experiment clearly demonstrated that the NINK score calculated with values comprising NK% and mRNA levels of NKp46 and IFN-γ in NK cells obtained even from blood stored for 1 day show good correlation with NK cell activity (**Figure 5**). When individuals were divided into groups comprising low and high NK cell activity or groups comprising low and high NINK score using the averages as cut-off values, most of the low NINK score group (87.5%) showed low NK cell activity, while most of the high NINK score group (85.7%) showed high NK cell activity. Taken together, these findings indicate that the NINK score is an effective measure of the natural cytotoxicity of specimens and obviates the need to assay for NK cell activity using target cells.

6. Discussion

Our demonstration indicates that the NINK score can be employed as a new index to determine the performance of natural cytotoxicity of PBMCs without the use of target cells or cell culture equipment for incubation. **Figure 6** shows the difference between the conventional index of NK cell activity and our new index of the NINK score. The conventional index of NK cell activity is useful since it can be determined by only using fluorescence or radioisotope-labeled target cells. However, it is often difficult to maintain good conditions of NK sensitivity in target cells. Technicians need to pre-culture cells under the same cell density and culture period (days) conditions in an effort to maintain good NK sensitivity of the cells. If daily measurement of NK activity is required, many pre-culture lines need to be prepared, which is unrealistic in a small institute. Additionally, since measurement of NK cell activity is based on a "bioassay", this traditional index is prone to variation between assays. This problem often troubles researchers since the altered sensitivity of target cells creates difficulties when combining results obtained from multiple assays. Similar to the measurement of NK cell activity by examining the release of ^{51}Cr or fluorescence labeling, the lactate dehydrogenase (LDH) release assay has a problem in terms of the bioassay. In the LDH release assay, the activity of LDH derived from lysed target cells in media is measured as an absorbance following incubation of effector cells with target cells [47–49]. As an alternative approach, the level of degranulation induced by stimulation with activating receptors was assayed to assess the lytic activity of NK cells *in lieu* of using target cells [50]. In this method, the increase in cell surface expression of LAMP1/CD107a is examined by flow cytometry following stimulation. The expression of LAMP1/CD107a is high on the membrane of lytic granules but increases on the plasma membrane as a result of degranulation. However, even this method is unable to avoid the use of incubated effector cells to assay the lytic activity, which can lead to difficulties resulting from the "bioassay". In contrast, determination of the NINK score does not require the use of target cells or cell culture equipment, and is therefore free of the potential complications associated

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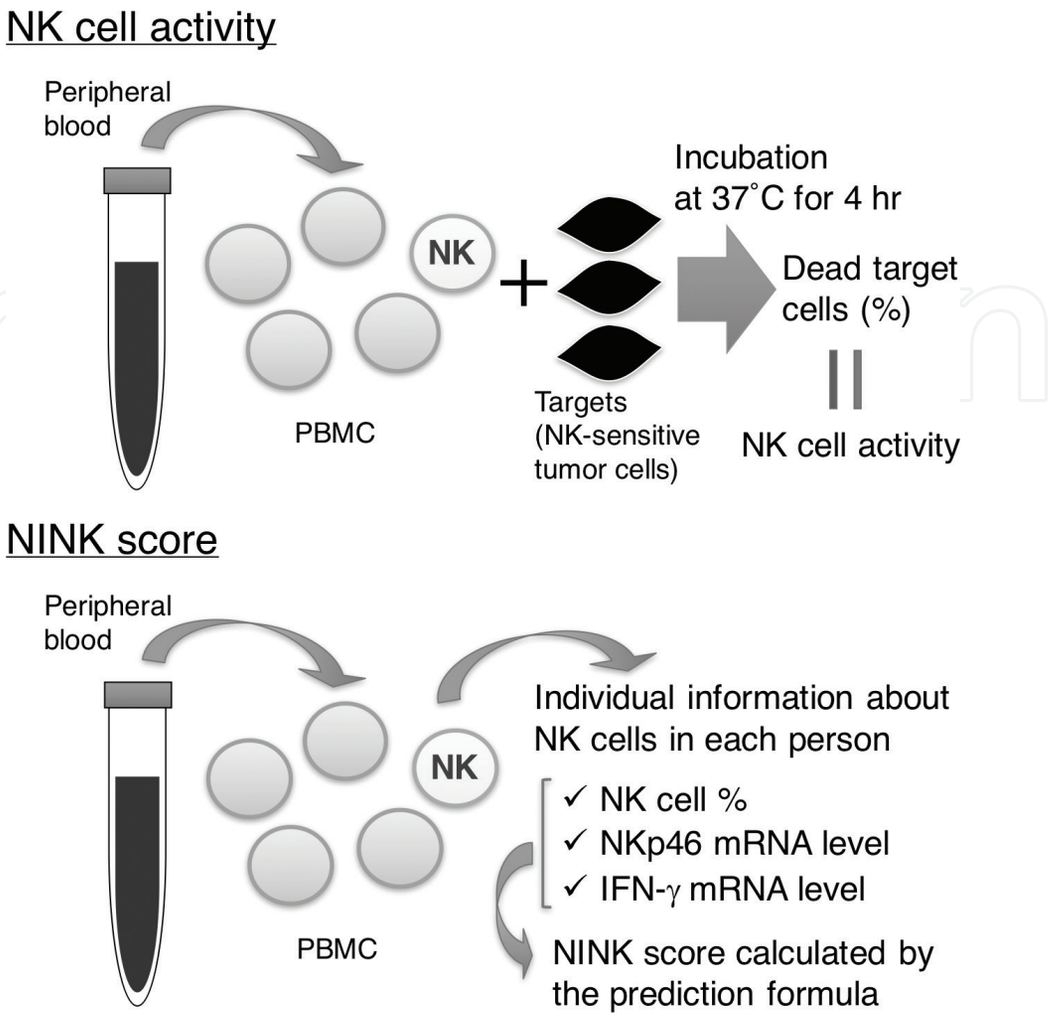

Figure 6. Illustration of differences between the conventional NK cell activity index and new NINK score index. To measure the NK cell activity, NK-sensitive target cells such as K562 cells need to be prepared and appropriately maintained prior to use in the assay, and then PBMCs need to be incubated with the target cells for 4 h, which is often troublesome and can cause large variation between assays. In contrast, a determination of the NINK score does not require the use of target cells or cell culture equipment for incubation. Technicians simply have to measure NK% and isolate NK cells from PBMCs. Messenger RNA levels of the two genes in the NK cells (retrieved from frozen storage) can then be determined and the NINK score calculated using the prediction formula with the measure values of the three parameters.

with the inappropriate preparation of those cells. Additionally, on any given day, technicians will measure NK%, isolate NK cells from PBMCs by flow cytometry or magnetically, and then store the NK cells frozen before subsequent assays are performed. At some later time, mRNA levels of NKp46 and IFN-γ can be determined in the frozen cells by real-time PCR and the NINK score calculated using the prediction formula with the measured values of the three parameters. Thus, employing our new approach based on the NINK score can free the technician from continuous pre-culture of target cells required in the traditional NK activity assay, and provides a stable tool to measure the performance of NK cells in each individual and independent bioassay. The NINK score is beneficial as a measure of NK cell performance in each individual, and is a requirement for clinicians working in

the field of cancer therapy as well as for the assessment of healthy individuals interested in preventive medicine and health promotion. Since NK cells are linked to the CTL response in acquired immunity as mentioned above, it may be valuable for clinicians to assess the progress of the functional status of NK cell activity in a patient following cancer therapy in a steady manner by using the NINK score. It would be better if the NK cell activity could be checked in terms of a solid parameter such as bone density or body fat percentage, which can be achieved by measuring the NINK score. A vast amount of data concerning the relationship between NK cell activity and various kinds of factors related to lifestyle can be collected using the NINK score, the results of which might lead to new insights concerning healthier and disease-preventive lifestyles from an immunological perspective. The ability to store isolated NK cell specimens temporarily in a frozen state provides a benefit in that the following measurements of mRNA levels can be performed simultaneously, which allows for an accurate comparison of NINK scores among specimens. Additionally, the NINK score may be useful as a screening device to identify NK cell-activating natural compounds from within a compound library using *in vitro* experiments of PBMC cultures. An actual effect of some compound on NK cell activity can then be examined using animal experiments (mice), even where a modified method of the NINK score may be utilized to measure the performance of NK cell activity *in lieu* of using the method with NK cell-sensitive murine targets such as YAC-1 cells [51, 52]. In contrast, the traditional method involved in the NK assay using target cells is unsuitable for these purposes given the lower stability resulting from the use of bio-assays as mentioned above. The NINK score might contribute to an assessment of the efficacy of a drug at different stages of cancer treatment in patients, as well as an assessment of the effectiveness of different lifestyles, exercise and food consumption in maintaining good health in individuals. We hope that the NINK score would be utilized in various fields to facilitate the promotion of general health in the population.

Acknowledgements

The authors thank Ms. Tamayo Hatayama, Shoko Yamamoto and Miho Ikeda for their technical help. The authors declare that there is no conflict of interests regarding the publication of this paper.

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