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Evaluation of a Flow Cytometry-Based Assay for Natural Killer Cell Activity in Clinical Settings

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Abstract

Natural killer (NK) cells are not only important in first line defence against viral and bacterial infections, but also in immune surveillance of malignant cells and thus NK cell cytotoxicity is primary indicator of immune function. Although chromium release assay is recognized as 'gold standard' for measuring NK cell activity, it has disadvantages like use of radioactive compounds, poor loading and high spontaneous release. It is difficult to perform this assay in clinical laboratory because of difficulties with disposal of radioactive waste and standardization problems. We describe a flow cytometry-based assay for the measurement of NK cell activity by incorporating fluorescent dye, DiO, into membranes of target cells. NK cell activity was measured at baseline, 1 and 4 weeks follow-up in 20 normal healthy individuals on a dietary supplement immunomodulator to enhance NK cell function. Mean baseline NK cell activity percentage (21.5; SD = 9.3) increased significantly to a maximum level at 1 week (31.3%; SD = 7.9; P = 0.007) and then returned to baseline level at 4 weeks (21.5; SD = 8.3). An important feature of flow cytometry-based assays is its ability to discriminate effector cells from target cells, and potential for explaining molecular interactions underlying target cell lysis. Under clinical settings, this assay will be of interest for frequently monitoring immunological status of patients on treatment for various diseases that affect their immune status. The assay is easy to perform without using radioactive material and thus could become a tool for monitoring pathogenesis and immune reconstitution.

Introduction

Cell-mediated cytotoxicity is a mechanism used by immune cells for defence against intracellular pathogens, tumour cells and allogeneic tissue grafts [1–3]. Natural killer (NK) cells are generally considered as components of innate immune defence, as they lack antigen-specific cell surface receptors [2, 4]. NK cells mediate a cell contact-dependent cytolysis of target cells, including those expressing foreign major histocompatibility complex (MHC) molecules. NK cells have been shown to participate in the early control of viral infection [1] and in immuno-surveillance [5] in humans and mice. Unlike cytotoxic T cells, NK cells are capable of inducing direct death of tumour and virally infected cells in the absence of specific immunization. Also, under many physiological and pathological conditions, NK cells have been identified as major producers of cytokines, such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin IL-10 [3, 6]. Target cell killing mediated by NK cells enhances the response of T cells either by decreasing the antigen load [7], or the debris created by NK cell-mediated killing might help in antigen presentation to cytotoxic T cells [8]. Even though most NK cells present in the body are in a resting state, once activated, their cytotoxic actions become vigorous.

One important and frequently measured indicator of immune function is NK cell cytotoxicity, which has been reported to be lower in certain conditions, such as primary immunodeficiency, late-stage HIV infections and pregnant women [4, 9–11]. NK cell cytotoxicity is significantly variable in apparently healthy individuals and can be categorized into a high or low level [12]. Recently, analysing NK cell numbers and function has become more of a routine practice in certain disease conditions. Also, a low level of NK cell activity may be useful in predicting patient outcome [10, 13].

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The 'gold standard' assay for NK cell activity has been the chromium release assay. Since 1968, the radioactive chromium (51Cr)-release method has been used to determine the cytolytic activity of effector cell populations. The chromium release assay has many limitations, such as: (1) hazardous radioactivity; (2) high cost; (3) short half-life; (4) increased staff requirements for radiation safety training and licensing; and (5) disposal of waste. The 51Cr method can only be used for a limited number of targets that immediately label with quantities adequate for definitive detection of lysis (i.e. the assay cannot be interpreted with high spontaneous Cr-release). Inter-laboratory variability in this assay is also of potential concern, especially as proficiency testing programs are not routinely in place. The final limitation of the assay is that death is not measured at a single-cell level. Owing to all of these reasons, the process is ongoing to develop and validate new assays that could replace the Cr-release, and several alternative methods have been introduced [14–17].

The purpose of the present study was to standardize and evaluate a flow cytometry-based assay in routine clinical laboratory settings for measuring NK cell activity. Flow cytometric assays prevent the problems related to the use of radioactivity and are rapid and more convenient for standardization.

We describe how flow cytometry can be used to determine NK cell activity over time in a group of individuals in a clinical trial. This assay uses two fluorescent dyes to discriminate between effector and target cells and between live and dead target cells. The first dye, 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) [18], is a green fluorescent dye, which was used to label the plasma membranes of K562, a human erythroleukaemic tumour cell line used as the target population. The second dye, propidium iodide (PI), a membrane impermeable, red fluorescent dye, was added during the assay, when targets cells membranes were disrupted by NK-like cells (PI bound to the DNA of cells with a compromised cell membrane). Of course, both membrane compromised targets and effectors were labelled by PI and exhibited red fluorescence; however, only targets prelabelled with DiO exhibited green fluorescence and were able to differentiate between effector and target cells. Intact target cells unaffected by effectors were single positive (and exhibited only green fluorescence), while targets killed by effectors bearing disrupted membranes were double positive (and exhibited green as well as red fluorescence). Our result indicated that this modified flow cytometric-based assay was highly reproducible and was able to differentiate people with low and high NK cell activity at baseline and also after the intake of an immunomodulating dietary supplement known to enhance NK cell activity. This assay was less time consuming and easily adaptable in a clinical laboratory setting.

Materials and methods

Subjects and samples. Healthy adults (n = 20) were recruited by referrals at the University of Miami Miller School of Medicine during 2010 after approval of the study by the Institutional Review Board. Subjects were given an immune-enhancing dietary supplement for 30 days. The dietary supplement used was Rice Bran Arabinoxylan Compound (RBAC) which is a nutritional supplement that has been shown to possess a biologic response modifier effect on immune system function in mice studies and also in humans [19, 20]. Venous blood was obtained at three different time points (baseline, 1 week and 4 weeks) from all participants and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation and cryopreserved at −140 °C.

Reagents. K562, human chronic myelogenous leukaemia cells (ATCC #CCL-243) were used for the NK assay. 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO) (Invitrogen, San Diego, CA, USA) was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, USA) to a concentration of 3 mM. Aliquots were frozen at −20 °C and thawed for each experiment. Roswell Park Memorial Institute (RPMI) media with and without phenol red were obtained from Gibco (Invitrogen). Complete medium (CM) consisted of RPMI medium supplemented with 10% foetal bovine serum, 100-U/ml penicillin, 100-mg/ml streptomycin and 50-mg/ml gentamicin. Propidium iodide (PI) (Sigma) was dissolved in CM to obtain a working solution of 10 mg/100 ml. Foetal bovine serum was obtained from Cellgro (Manassas, USA).

Flow cytometric enumeration of NK cell subsets was carried out using antibodies against CD45, CD3 and $CD16^+$ $CD56^+$. All antibodies were purchased from BD Biosciences, CA. Flow cytometry-based assay for NK cell activity was modified from previously published methods for measuring NK activity [18, 21].

Preparation of target cells. Log phase cultures of K562, human chronic myelogenous leukaemia cells (ATCC #CCL-243) were re-suspended at a concentration of 10^6 cells/ml in phosphate-buffered saline (PBSA) and labelled by adding 2 μl of 3 mM Dioctadecyloxacarbocyanine perchlorate (DiO)/ml of K562 cells, incubated at 37 °C in 5% CO_2 incubator for 20 min, and washed and re-suspended to a final concentration of 10^6 cells/ml in RPMI-1640 with 10% foetal calf serum (R-10) medium without phenol red.

Effector cell preparation. The PBMCs were assayed at the same time to avoid any inter-assay variations. Thawed, overnight rested PBMCs were washed and re-suspended to a final concentration of 10^6 cells/ml in R-10 medium without phenol red.

NK Cell Activity Assay. Effector and target cells were added in tubes to create four different effector-to-target (E:T) ratios from 25:1, 12.5:1, 6.25:1 and 3.125:1.

A solution of 0.15 mM PI (130 μl) was added to the tubes and centrifuged for 30 s at 1000 *g* to pellet the cells and was further incubated for 2 h at 37 °C in 5% CO_2 incubator and analysed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A total of 20,000 events were acquired. Data were analysed using CELLQUEST pro software (v5.2) (BD Biosciences). Controls consisted of target cells only plus PI and effector cells only plus PI and were used to detect spontaneous lysis and nonviable effector cells, respectively.

Calculation of per cent lysis, lytic units and lytic activity. Per cent lysis was calculated as [Cells positive for both DiO and PI/Total DiO labelled cells] * 100 − spontaneous lysis.

Lytic units. The lytic unit was defined as the number of effector cells required to lyse a specified percentage of target cells [22]. The reference lysis level was considered as 15%. Lytic units = E:T ratio that causes 15% lysis * number of targets.

Lytic activity. Lytic activity was defined as the number of lytic units contained in 10^7 effector cells (Number of lytic units/10^7 effectors = 10^7/T*Xp (T, number of target cells; p, the reference lysis level (15%); Xp, E:T ratio required to lyse % of the targets)). Lytic units were calculated as the number of effector cells required to lyse 15% of 2×10^4 target cells with the results being expressed as the number of lytic units contained in 1×10^6 PBMCs. Per cent specific lysis for 15% target cells at each E:T ratio was calculated. Per cent specific lysis was then used to calculate the lytic units.

Statistical analysis. Natural killer cell activity was evaluated with paired samples *t*-tests between the baseline, 1-week and 4-week time points.

Results

Frequency and absolute numbers of NK

Frequency and absolute numbers of NK cells did not change after intake of immunomodulating dietary supplement. A representative dot plot showing flow cytometric staining for NK cell subsets from peripheral blood is depicted in Fig. 1A. Lymphocytes were gated as $CD45^+$ cells, and NK cells were further identified as $CD45^+$ CD3-$CD16^+$ $CD56^+$ cells. The frequencies (Fig. 1B) and absolute numbers (Fig. 1C) of NK cells did not change after immune modulator intake in healthy adults (Fig. 1) at any of the time points tested.

Per cent specific NK cell activity

Per cent specific NK cell activity at different E:T ratios at baseline, 1 week and 4 weeks after intake of immunomodulating dietary supplement was consistent for each time point. As shown in Fig. 2A,B, NK cell activity was clearly evident with different E:T ratios at different time

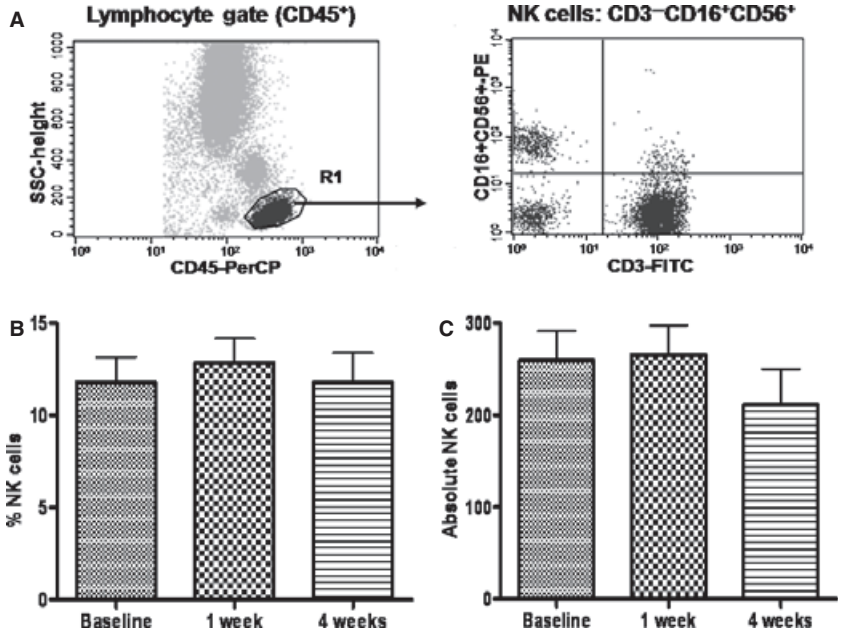

Figure 1 Frequency and absolute number of NK cells during intake of dietary supplement: Frequency and absolute number of NK cells did not change after dietary supplement intake in healthy adults. Whole blood (100 μl) was stained with antibodies against CD45, CD3 and $CD16^+$ 56 for 30 min in room temperature. After lysis of RBC, cells were washed, fixed and analysed by flow cytometry. (A) Lymphocytes were gated as $CD45^+$ cells and NK cells were further identified as $CD45^+$ CD3-$CD16^+$ CD56+ cells. Bar chart showing (B) Mean frequencies and (C) Mean absolute numbers of NK cells at baseline and 1 week and 4 weeks after dietary supplement intake. Each bar represents the mean value and error bars are depicted based on the standards error of mean value of each time point.

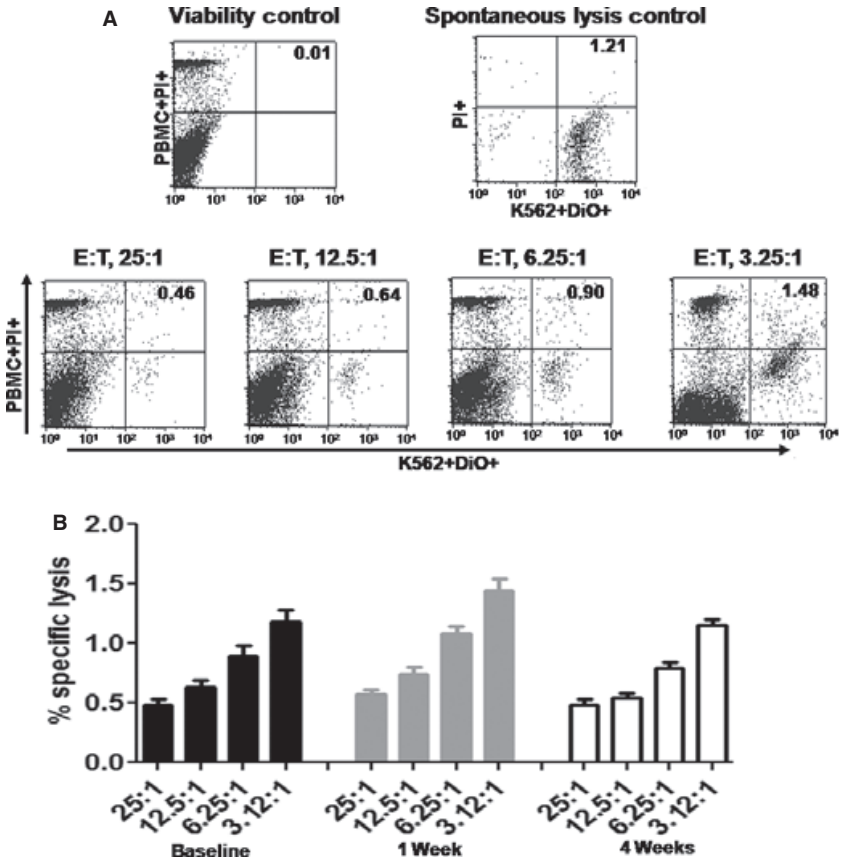

Figure 2 Flow cytometry-based NK cell activity assay: Effector (PBMC) and 10,000 target cells (K562 cells labelled with DiO) were added to tubes to create four different effector-to-target (E:T) ratios from 25:1, 12.5:1, 6.25:1 and 3.125:1. A solution of 130 μl of (0.15 mM) propidium iodide (PI) was added to tubes and incubated for 2 h at 37 °C in 5% CO_2 and analysed by flow cytometry. A total of 20,000 events were acquired. Controls consisted of target cells only plus PI and effector cells only plus PI and were used to detect spontaneous lysis and nonviable effector cells, respectively. (A) Representative dot plots showing E:T ratios at 25:1, 12.5:1. 6.25:1 and 3.125:1 along with viability control which had only PBMCs with PI and spontaneous lysis control, which had only K562 with DiO and PI. (B) Each bar represents the mean representing mean per cent specific lysis at 25:1, 12.5:1. 6.25:1, 3.125:1 for baseline, 1 week and 4 weeks time point and error bars are depicted based on the standards error of mean value of each time point.

points. Maximum NK cell activity was observed at 1 week compared to other time points.

Natural killer cell activity

Changes in NK cell activity after intake of immunomodulating dietary supplement were detectable by the flow-based assay. Representative dot plots showing flow cytometric determination of NK cell activity of one participant are depicted in Fig. 3A. Changes in NK cell activity from baseline to 4 weeks were measurable by this assay. During the intake of the immunomodulating dietary supplement, baseline mean percentage NK cell activity was 21.5 ± 9.3, which increased significantly at 1 week (31.3 ± 7.9, P = 0.007) and then declined to its baseline level at 4 weeks (21.5 ± 8.3; Fig. 3B).

Per cent specific lysis and lytic units determination

As a second index of NK cell activity, lytic units were calculated. We calculated the lytic units per PBMC and the lytic units obtained also showed the similar pattern as NK cell activity. Per cent specific lysis for 15% target cells were higher at 1 week (Fig. 3C), indicating a clear difference in the per cent specific lysis following intake of the dietary supplement. Lytic units obtained at baseline, 1 week and 4 weeks after immune modulator intake for individual study participants are given in Table 1. Mean lytic units at 1 week were 168.2 ± 68.9, which were significantly higher than that of baseline (115.7 ± 56.8; P = 0.017) levels. The lytic units declined to near baseline levels at 4 weeks (95.5 ± 44.4; P = 0.008) (Fig. 3D). No significant changes were observed in the absolute number of NK cells.

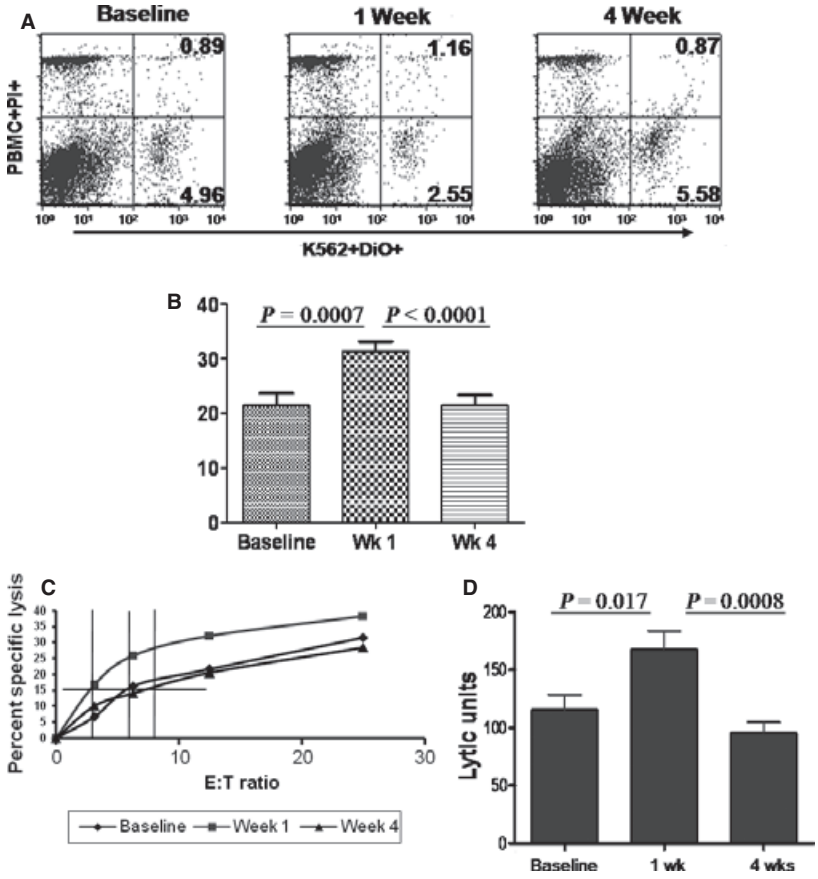

Figure 3 Per cent NK cell activity and lytic units after 1 week of dietary supplement intake: (A) Representative dot plots showing flow cytometry-based NK cell activity at baseline, 1 week and 4 weeks time points in a study participant. (B) Percentage of NK cell activity recorded for samples at baseline, 1 week and 4 weeks time points and (C) Line graph showing per cent specific lysis for 15% target cells at different E:T ratios for baseline, 1 week and 4 weeks time points of a study participant. (D) Bar chart showing lytic units calculated based on per cent specific lysis for 15% target cells for samples at baseline, 1 week and 4 weeks time points. Each bar represents the mean value and error bars are depicted based on the standards error of mean value of each time point.

Discussion

Natural killer cells are important components of the innate immune system, owing to their cytokine production and cytolytic activity against target cells. NK cells appear to play a crucial role in innate immunologic defence and regulation of immune response in a non-MHC–restricted manner [3–5]. In clinical settings, *in vitro* NK cell activity has been an important predictor of possible correlations between NK cell activity and disease outcome or progression [2, 3, 5, 23, 24]. The 'gold standard' assay for NK cell activity has been the 51Cr-release assay. In this assay, internalized radioactive chromium released by target cells upon cytolysis by NK cells were quantified by measuring the amount of radioactivity released using a gamma counter [10, 18, 25, 26]. However, innumerable difficulties with this assay prevent it from being routinely used in clinical settings. It is difficult to standardize, expensive, time consuming, uses reagents with short half-lives, and requires disposal of radioactive and hazardous materials. Therefore, development of an alternative method for measuring NK cell activity would be desirable in clinical settings. Under clinical settings, a flow cytometry-based NK cell activity assay would be of interest for frequently monitoring the immunological status of patients on treatment for different diseases [21]. Advantages of this approach include: (1) no use of radioactive materials; (2) performed easily using large number of samples; and (3) multiple time points can be performed together to reduce intra- and inter-assay variability [18, 21, 25, 26]. In the current study, flow cytometry-based NK cell activity was highly reproducible, and these flow data could be used for calculating lytic units, which are conventionally used for expressing the NK cell cytotoxicity.

In this study, we selected a group of 20 healthy individuals who were taking a dietary supplement known to enhance NK cell activity. We assessed NK cell activity over three time points by a modified flow cytometry-based approach described previously [18, 21]. Our

Table 1 Lytic units at baseline, 1 week and 4 weeks during intake of a dietary supplement.

Subject	Baseline LU	1 week LU	4 weeks LU
1	161	250	80
2	108	161	124
3	156	238	111
4	99	357	64
5	172	100	63
6	113	52	113
7	83	138	100
8	42	93	93
9	89	192	71
10	20	156	85
11	208	64	52
12	42	208	16
13	54	179	63
14	50	102	46
15	208	147	156
16	156	185	192
17	119	200	104
18	100	156	119
19	172	185	111
20	161	200	147
Mean ± SD	115.66 ± 56.79	168.17 ± 69.89*	95.486 ± 41.44

*Mean lytic units at 1 week were significantly higher than that of baseline (P = 0.017) levels.

approach was to standardize the test and explore the feasibility of this assay in our clinical lab setting. We selected normal healthy subjects for the following reasons. First, as these individuals were healthy, the outcomes of the data reflect the real effect of the dietary supplement on NK cells, and the detected changes in the data can be compared between pre- and post-immune enhancement. Second, the minor variations in the data are a reflection of immune enhancement owing to the dietary supplement in a group of subjects whose immune system is in a resting state. We did not compare our data with classic 51Cr-release, as we assessed the outcomes before and after the intake of the dietary supplement. Thus, the results of the flow-based data performed simultaneously reflected the actual changes in the NK cell activity owing to effect of the dietary supplement. Unlike other studies using a flow-based approach, our sample of subjects enabled us to validate the results of the assay by simultaneously analysing the NK cell activity in the same individuals before and after immunomodulation.

Many reports have documented flow cytometry-based methods for measuring NK cell activity [10, 18, 26, 27]. The method described here utilized the incorporation of a green lipophilic fluorescent dye, DiO, into the membranes of target cells grown in culture. This dye allows the easy discrimination of target cells from effector cells by flow cytometry. In addition, by incorporating PI, dead cells could be identified by the PI nuclear DNA intercalation [18]. As PI fluoresces in the orange-red spectrum, the percentage of target cells that were damaged by the effector NK cells could be accurately determined by dual-fluorescence analysis.

This method utilizes components of previously published flow-based work [18, 21] but we tested this approach in a clinical laboratory setting with samples of subjects in whom enhancement of NK cell activity was expected as they were normal healthy humans without any clinical conditions which affects the immune system. We included both target and effector cells in analysing NK cell activity, rather than only utilizing target cells. This approach allows the consideration of changes in scatter properties of less distinct effector and target populations, as cells become damaged. In our analysis, we defined target cells as those retaining bright DiO staining (double fluorescent). We reasoned that NK cells may also show weakened membrane integrity following incubation with PI and that the per cent lysis of targets might be overestimated if cells that were stained with PI but not with DiO were included. Likewise, the total target population was used as the denominator, when determining the per cent lysis by NK cells. The choice of DiO has an advantage in terms of emission spectra, as DiO labelled cells quickly and efficiently and do not leak appreciably within the time frame of the flow cytometric assay [10, 25].

We also successfully utilized the data derived from this assay for the calculation of lytic units. The lytic unit is principally used to express levels of NK cell activity. Lytic units can be considered a second index of cytotoxic activity [22]. Lytic units were calculated as the number of effector cells required to lyse 15% of 2×10^4 target cells, expressed as the number of lytic units contained in 1×10^6 PBMC. Other than using raw cytotoxicity data, the lytic unit has been the most common method of presenting data in human and animal tumour immune studies involving NK cells, lymphokine-activated killer cells and cytotoxic T cells [22, 28]. We observed that our lytic unit data were comparable with that of NK cell activity, indicating that changes observed by this assay reflected actual changes.

In the clinical setting, reporting NK cell activity in lytic units would be beneficial, as clinicians could analyse the data with different perspectives to maximize their usefulness with minimum variability. The assay results calculated as the per cent specific lysis at the four different E:T ratios can be converted into lytic units, as commonly carried out for the 51Cr-release assay. It is also possible that the cytotoxicity data can be presented on a per effector cell basis, using the absolute cell number values determined in the assay (LU/absolute number of NK cells or PBMC). This is advantageous for discrimination between individuals with few NK cells, but strong NK cell activity from those with many NK cells, but weak NK cell activity. Further, like the 51Cr-release assay, the

flow-based assay can be used in clinical settings, as it discriminates patients with underlying immunodeficiency, whose NK cell activity may be depressed [21]. Our data also showed that the assay is sensitive enough to measure biologic variations, as indicated by changes observed in the NK cell activity of individual participants serially tested over time and/or as result of immunomodulation.

Recent discovery of inhibitory and activating receptors on NK cells has brought increasing interest in NK cell biology and activity in disease settings [29–31]. NK cells are programmed to interact with tissue or hematopoietic cells and play a major role in regulating innate immunity [5, 32]. The phenotypic and functional heterogeneity of NK cell subsets re-focused attention on their lytic activity and the molecular interactions between effectors and targets. The new multi-parameter flow cytometry-based assays offer an opportunity for simultaneous measurement of NK phenotype and NK cell activity of various NK subsets. The assay described here could be adapted to include simultaneous measurement of more phenotypic and functional markers of NK cells along with NK cell activity through a multi-parameter approach. An additional advantage is that the assay is non-radioactive. The flow cytometry-based assay used in this study can reproducibly be used as a routine NK cell activity assay in clinical laboratory settings, replacing the 51Cr-release assay and could also provide data on NK cell activity per absolute number of NK cells or PBMC present in the sample.

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