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Measuring natural killer cell cytotoxicity by flow cytometry

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Summary

Natural killer (NK) cell cytotoxic function is critical in guarding an organism against viral infections and malignantly transformed cells. Although the ^{51}Chromium (^{51}Cr)-release assay is regarded as the gold standard for assessing NK cell cytolytic activity, this method is associated with a number of technical problems including the use of radioactive reagents and inconsistent assay performance, due to the lack of assay standardisation across laboratories. Here we describe the setup of a flow cytometry (FC) based method for the measurement of NK cell cytotoxicity, suitable for patient testing. The FC protocol was assessed using four normal samples, and reference values for NK activity of the local Hong Kong population were defined by 40 peripheral blood samples from healthy volunteers. For method validation, we tested a total of 13 specimens including nine healthy individuals and four patients with clinical conditions that were expected to have NK cell dysfunction. We directly compared those results between FC and the ^{51}Cr-release assay and we were able to demonstrate that FC is a clinically valid method for measuring NK cell function in a clinical setting.

Key words: Natural killer cells; cytotoxicity; NK function; flow-cytometry; chromium.

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INTRODUCTION

Defective natural killer (NK) cell function is the cause of a number of primary immunodeficiency diseases (PIDs).[1–3] Patients with PIDs often suffer from increased susceptibility to infections and malignancies,[4] and early detection of NK cell deficiency plays a critical role in both the diagnosis and management of these disorders.[5] Haemophagocytic lymphohistiocytosis (HLH) is a life-threatening PID characterised by excessive activation of macrophages and cytokine production. Cytotoxic defects in NK cells can be found in most HLH patients,[3,6–9] so laboratory tests assessing NK cell function have an important clinical utility for such diseases. One way of identifying HLH is by screening for mutation(s) within relevant genes that are involved within the NK cell cytotoxicity pathway, but the DNA sequencing method has the downsides of being time-consuming and labour intensive. On the other hand, NK cell functional assay provides direct insights into the cytotoxic machinery and thus can guide the diagnostic work-up, and help in formulating the search for genetic disorders. Hence, functional analysis can be a powerful approach for investigating NK cell function deficiency.

The ^{51}Chromium (^{51}Cr)-release assay has traditionally been considered to be the gold standard for measuring NK cell cytotoxic function. Briefly, target cells are loaded *in vitro* with radioactive chromium and lysis is determined by measuring the radioactivity emitted by the chromium released by dying cells in the supernatant. Although this is a widely used method for measuring T or NK cell cytotoxicity, numerous disadvantages are associated with this technique including laborious and lengthy procedures, low sensitivity, poor cell labelling, high spontaneous release of radioisotope from the target cells, and biohazardous material.[10–12]

In recent years there have been a number of publications that have demonstrated excellent reliability using flow cytometry (FC) to measure NK cell cytotoxicity.[10,11,13–17] FC is considered a better method because it does not require the manipulation of radioactive isotopes, has a fast turnover time but, most importantly, shows much greater sensitivity because it allows cytolysis assessment at the single-cell level. The primary goal of this report is to refine an in-house protocol of FC for determination of NK cell cytotoxicity suitable for patient service in our laboratory.

The main procedures used in our FC method for measuring NK cell cytotoxicity can be briefly generalised as: (1) isolation of peripheral blood mononuclear cells (PBMC); (2) labelling of target K562 cells with DiO dye; (3) cell concentration adjustment of the isolated PBMC based on NK cell percentage in the sample; (4) incubation of adjusted unfractionated PBMC with the labelled target cells; (5) counterstaining of dead cells by 7AAD post-incubation; and (6) analysis by FC. This report details the performance comparison between the ^{51}Cr-release assay and FC for measuring NK cytotoxicity, optimisations, determination of reference values for the local population in Hong Kong and the validation of FC by testing patients with known defective NK cell cytotoxic function.

MATERIALS AND METHODS

Sample selection

Samples collected from four healthy adults were used for method evaluation and optimisations, while samples collected from 40 volunteers were used for establishing the local (i.e., Hong Kong) reference values. Samples from four patients expected to have defects in NK cell function (referred by the Pediatrics Unit, Queen Mary Hospital, Hong Kong), and nine healthy individuals were collected for validation of FC. This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 18–427).

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Specimen requirement

A sample of 3 mL EDTA blood (used for enumeration of NK cell numbers by FC) and another of 10 mL heparinised blood was required. Samples must have been processed within 2 hours of collection and partially clotted samples were rejected.

Target cell labelling

K562 was used, a non-adherent and rounded myelogenous leukaemia cell-line (ATCC, CCL-243) commonly used as target cells in NK cytotoxicity assays. The cell-line was cultured in RPMI (GIBCO, Life Technologies, USA) supplemented with 10% FBS.

For FC, K562 cells were washed twice and resuspended with PBS. Cell concentration was adjusted to 0.75×10^6 cells/mL in a total volume of 2 mL, followed by addition of 1.8 µL DiO dye (Vybrant DiO Cell-Labeling Solution, #V22886; ThermoFisher, USA) to the 2 mL mixture and this was immediately vortexed. We found this volume provided the most effective DiO labelling. The mixture was incubated in a 37°C water bath for 5 min. After incubation, the labelled cells were washed twice with PBS and resuspended in 10% FCS RPMI. The labelled K562 cells were adjusted to a final concentration at 1×10^5 cells/mL (or otherwise stated) and kept in the dark until ready for use.

For the ^{51}Cr-release assay, the target K562 cells were labelled for 45–60 min with 100 µCi of 6 mCi/mL ^{51}Cr-sodium chromate ($Na_2{}^{51}CrO_4$; NEZ03001MC; Perkin-Elmer, USA) per approximately 2×10^6 target cells. ^{51}Cr-labelled target cells were suspended in 10% FCS RPMI at 1×10^5 cells/mL.

Effector cell preparation

For the extraction of PBMC, we layered 10 mL heparinised blood onto 8 mL Lymphoprep (#7546999; Beckman Coulter Diagnostics, USA) followed by centrifugation at 930 × *g* for 20 min (without break). The PBMC were collected between the interphase. The PBMC were washed once with PBS and cells were resuspended in 1.1 mL 10% FCS RPMI. The cell concentration of the mixture was counted and set aside. Concurrently, using a separate EDTA blood sample, the NK cell percentage was determined using the lymphocyte subset profile by 4-colour flow cytometry. The NK cell percentage was used for calculating the volume adjustment required of the unfractionated PBMC mixture, so that only fixed numbers of NK cells were tested in the assay.

Effector/target cells incubation

We added 0.1 mL of unfractionated PBMC to 0.1 mL labelled target cell suspension at predetermined 2-fold serial dilution effector/target cell ratios (e.g., 16:1, 8:1, 4:1, 2:1, etc.). The cell mixtures were kept at 37°C in the CO_2 incubator for 4 hours to allow enough time for cytolytic activity to progress. When the incubation was complete, the following was carried out for each method.

For FC, 5 µL of 10-fold diluted 7AAD (#A1310; ThermoFisher) was added to all tubes and placed at 37°C in the CO_2 incubator for 10 min. FC analysis was performed immediately after incubation with DiO+ target cells gated (ex/em 484/501), and the live/dead cell percentages visualised by 7AAD exclusion (ex/em 488/647). At least 5000 DiO+ cells were collected.

For the ^{51}Cr-release assay, the samples were centrifuged for 5 min at 470 × *g* to pellet cells to the bottom of the tube, and 25 µL of the supernatant from each well was carefully harvested and added to wells of a Luma-96 plate (Perkin-Elmer) for measurement of radioactivity. The supernatants were allowed to dry in a fume hood overnight and gamma counts per minute (cpm) were measured using a Topcount NXTTM microplate scintillation and luminescence counter (Packard Bioscience, USA). The corrected percent lysis for each concentration of effector cells was calculated using the mean cpm for each replicate of wells: % specific lysis = 100 × [mean sample ^{51}Cr-release (cpm) – mean spontaneous ^{51}Cr-release]/[mean maximum ^{51}Cr release (cpm) – mean spontaneous ^{51}Cr-release (cpm)]. Maximum ^{51}Cr-release was determined by target cell lysis with Triton X-100.

Statistical analysis

Data were analysed using GraphPad Prism v5.04 software (GraphPad Software, USA). All results were considered significant at p values <0.05. Repeated measures 2 way ANOVA tests were used to compare performance of the FC method with the ^{51}Cr-release assay. The Sidak's multiple comparisons test was performed to determine the level of statistical significance between the FC method and the ^{51}Cr-release assay at specific effector-to-target ratios. Reference values for the local population in Hong Kong were defined by the lower fifth percentile, at each NK-to-target ratio at 16:1, 8:1, 4:1 and 2:1.

RESULTS

FC was able to detect higher level of NK cell cytotoxicity than the ^{51}Cr-release assay

We compared the level of NK cell cytotoxicity between the ^{51}Cr-release assay and FC by utilising PBMC collected from four healthy volunteers. An example of NK function activity measured by FC is shown in Fig. 1. For simplicity, only the number of total PBMC was adjusted (i.e., the number of NK cells at each effector-to-target ratio differed between subjects). We started measuring the levels of NK cell cytotoxicity at effector-to-target ratios from 80:1 and 10:1 for the two methods and the results are shown in Table 1. We noted that the trend was not linear and the cause was unclear, possibly due to suboptimal preparation of the serial dilutions, or NK cytotoxicity at those PBMC-to-target ratios were simply non-linear in nature. Despite this, on average, FC was able to obtain a significantly higher level of NK cytotoxicity (greater by 12.4–20.5%) than the ^{51}Cr-release assay at all effector-to-target ratios measured (Fig. 2) (p<0.01). These data suggest that the analytical sensitivity of FC is at least equal, if not better than the traditional ^{51}Cr-release assay for measuring NK cell cytolytic activity.

Because FC was able to measure high levels of NK cell cytotoxicity at PBMC-to-target ratios starting at 80:1, we repeated the experiment but lowered the number of PBMC by 10-fold (i.e., PBMC-to-target ratio 8:1, 4:1, 2:1 and 1:1) to see if we could continue to obtain high levels of NK cell cytotoxicity that would warrant a lower volume of blood sample required for the assay (Table 2). After reducing the number of PBMC, the levels of NK cell cytotoxicity for all subjects expectedly dropped by at least 10 times at all PBMC-to-target ratios (i.e., 8:1, 4:1, 2:1, 1:1) for either FC or ^{51}Cr-release assay (Fig. 3). However, the difference between the two methods also became less apparent and statistically insignificant. Nevertheless, it appeared that FC remained able to measure higher levels of NK cell cytolytic activity even with a reduced number of PBMC per target (at least at 8:1 and 4:1 ratios), most probably due to FC having greater analytical sensitivity than the ^{51}Cr-release assay.

To potentially facilitate a speedier workflow, we explored the effect of reducing the incubation time from 4 h to 2 h. The levels of NK cell cytotoxicity with these adjustments at various effector-to-target ratios are shown in Table 3. Because the level of NK cell cytotoxicity was at least three times higher at 4 h incubation than at 2 h in all four subjects measured, we decided to retain the incubation time of 4 h in subsequent experiments as it enhanced the level of NK cell cytotoxicity that could be measured.

Establishing the local reference values for FC

Using PBMC without adjusting for NK cell numbers may not be very useful in a clinical setting, since the low level of NK cell cytotoxicity could be due to either a defect in NK cell cytotoxic machineries or low NK cell number to elicit effector function. For the purpose of allowing more

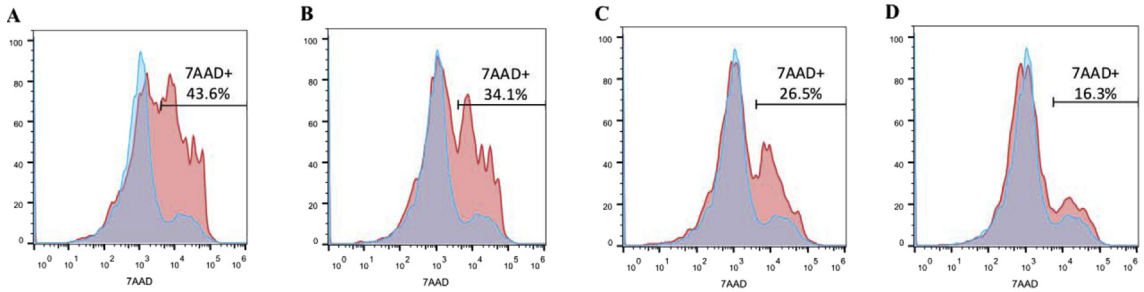

Fig. 1 NK cell cytotoxicity measured by flow cytometry. Effector-to-target ratios at (A) 16:1, (B) 8:1, (C) 4:1 and (D) 2:1. The level of NK cell cytotoxicity decreased as the effector-to-target ratio was reduced. Blue: K562-only spontaneous cell death (i.e., background). Red: K562 incubated with PBMC.

Table 1 Comparison of NK cell cytotoxicity (%) measured by flow cytometry (FC) and ^{51}Cr-release assay

PBMC-to-target			NK cytotoxicity (%)							
			80:1		40:1		20:1		10:1	
Sample	%NK	#NK @ 80:1	FC	^{51}Cr	FC	^{51}Cr	FC	^{51}Cr	FC	^{51}Cr
1	17.4	13.9	85.4	76.4	86.8	63.3	82.2	63.5	70.9	41.8
2	8.6	6.9	76.0	67.4	71.8	67.4	68.0	63.1	61.1	47.1
3	8.7	7.0	83.6	64.3	83.5	67.8	81.2	54.0	73.7	48.7
4	19.1	15.3	82.9	70.3	81.3	62.1	72.6	56.3	60.3	46.3
		Mean	82.0	69.6	80.9	65.2	76.0	59.2	66.5	46.0

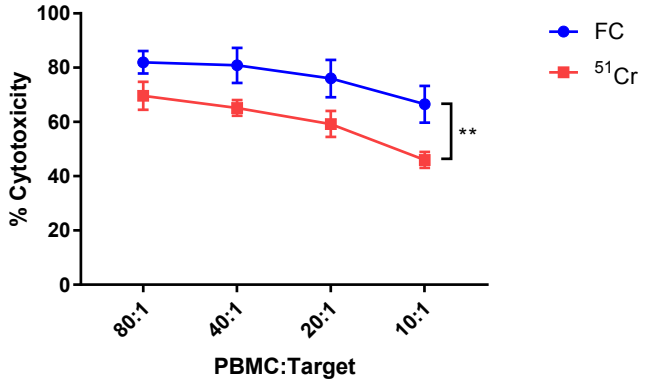

Fig. 2 Flow cytometry was able to detect a significantly higher level of NK cell cytotoxicity than the ^{51}Cr-release assay (n=4). Means with SD. Repeated measures 2 ways ANOVA.

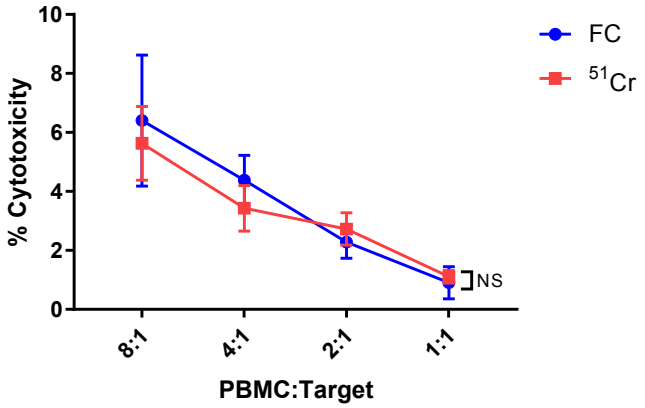

Fig. 3 Levels of NK cell cytotoxicity became indistinguishable between flow cytometry and the ^{51}Cr-release assay at low PBMC-to-target ratios (n=4). Means with SD. Repeated measures 2 ways ANOVA.

comprehensive clinical interpretation for NK cell function, especially to enable the comparison between subjects, we established cut-off values using unfractionated PBMC with a fixed number of NK cells. Adjusting the number of unfractionated PBMC with a known percentage of NK cells could set the absolute number of NK cells for the assay. Based on earlier data, we considered 16 NK cells per one K562 cell target (followed by two-fold dilutions) to be an appropriate starting effector-to-target ratio for establishing our reference values. A total of 40 healthy adult subjects were measured to establish the local reference values at predetermined effector-

Table 2 NK cell activity (%) measured by flow cytometry (FC) and ^{51}Cr-release assay with reduced number of PBMC

PBMC-to-target			NK cytotoxicity (%)							
			8:1		4:1		2:1		1:1	
Sample	%NK	#NK @ 8:1	FC	^{51}Cr	FC	^{51}Cr	FC	^{51}Cr	FC	^{51}Cr
1	18.2	1.5	9.6	7.4	5.2	4.4	1.9	2.6	0.5	1.4
2	15.2	1.2	4.5	4.7	5.0	3.6	3.0	2.6	1.7	0.9
3	10.3	0.8	6.0	4.8	3.7	2.6	2.4	3.5	0.8	1.0
4	22.4	1.8	5.5	5.6	3.6	3.1	1.8	2.2	0.6	1.1
		Mean	6.4	5.6	4.3	3.4	2.3	2.7	0.9	1.1

Table 3 Level of NK cell cytolysis (%) measured by flow cytometry at 2 or 4 hours post-incubation

PBMC-to-target (K562)			NK cytotoxicity (%)							
			8:1		4:1		2:1		1:1	
#	%NK	#NK @ 8:1	2 h	4 h	2 h	4 h	2 h	4 h	2 h	4 h
1	18.2	1.5	2.3	9.6	1.0	5.2	0.6	1.9	0.0	0.5
2	15.2	1.2	1.8	4.5	0.6	5.0	0.0	3.0	0.0	1.7
3	10.3	0.8	1.6	6.0	0.9	3.7	0.4	2.4	0.6	0.8
4	22.4	1.8	2.5	5.5	1.9	3.6	1.0	1.8	0.6	0.6
		Mean	2.0	6.4	1.1	4.3	0.5	2.3	0.3	0.9

to-target ratios (Supplementary Table 1, Appendix A). Calculating the 5th percentile of the pooled data generated the critical values that would allow discrimination of NK cell cytotoxicity dysfunction.

Validation of FC by patient testing

To validate FC for detecting defects in NK cell cytolysis and to determine FC's clinical sensitivity and specificity, we tested a total of 13 individuals by both FC and the ^{51}Cr-release assay. Amongst these 13 samples, four were referred by the paediatric units from patients that had previously been diagnosed with HLH or confirmed with NK dysfunction elsewhere; thus, all were expected to have low NK cell activity (Table 4). The other nine samples were healthy controls. All of the healthy controls were found negative for NK cell defects (i.e., normal) by both assays, indicating good diagnostic specificity in either methodology (Fig. 4). Interestingly, only FC was able to detect all four positive cases (i.e., NK cell activity below reference values); the ^{51}Cr-release assay could only identify Patients 1, 3 and 4, suggesting the superior diagnostic sensitivity of FC. The medical history of Patient 2 revealed no significant difference in the NK cell percentage, absolute counts or other information that could explain the better diagnostic sensitivity of FC with this patient. Nevertheless, because the sample size was limited as PIDs are rare, whether the diagnostic sensitivity of FC was truly superior would need further verification when other suitable clinical samples become available in the future.

DISCUSSION

NK cells are a major component of innate immunity and are responsible for immune surveillance. This arm of immunity induces direct cytotoxicity and the secretion of cytokine without the need of recognising specific antigens presented by the major histocompatibility complex molecules. Notably, NK cytotoxicity is clearly a major defense mechanism against infections, malignancies, and its malfunction is involved in various pathological diseases. Being able to measure NK cell cytotoxic activity has important clinical implications and NK cell function analysis has become increasingly demanded as a part of the clinical service. The ^{51}Cr-release assay has long been considered the gold standard for determining NK cell activity, despite a number of technical drawbacks such as the generation of radioactive waste and spontaneous leakage of ^{51}Cr from target cells that could compromise analytical sensitivity. Thus, the development of an alternative method to replace the ^{51}Cr-release assay for a routine clinical service is becoming increasingly desirable. FC featured several superior advantages in contrast to the ^{51}Cr-release assay, with the most prominent being eliminating the use of radioactive reagents, thereby greatly improving laboratory safety and removing the need for radioactive work licensing and waste disposal. In addition, once set up, the long term running and reagent costs of FC are less costly, especially because ^{51}Cr only has a half-life of 28 days and requires regular replacement which can be inconvenient. Most importantly, a number of reports have previously documented FC to be an effective method for detecting NK cell cytotoxicity with performance comparable to the ^{51}Cr-release assay.[15,18–22] The aim of this study was to refine a FC-based assay for measuring NK cell cytotoxicity that would be suitable for patient testing. We were able to validate the FC assay and we correctly distinguished subjects expected to have abnormally low levels of NK activity from healthy individuals. Although our sample size for validation was limited due to the rarity of positive cases, our evaluation demonstrated findings that are consistent with earlier reports, which suggest that FC is as reliable and has equal to better diagnostic sensitivity than the ^{51}Cr-release assay in measuring NK cell cytolysis.[18,23,24] Nevertheless, given that the number of positive samples were restricted, whether FC truly has better sensitivity will need to be further verified when more clinical samples become available in the future.

The ^{51}Cr-release assay in our laboratory utilises unfractionated PBMC without adjusting the number of NK cells. So, when low NK cell activity was obtained, it was unclear whether the results were due to a low number of NK cells in the PBMC, or dysfunction with the NK cell cytotoxic machineries. In order to make interpretation easier, reference values of our FC method were collected by using unfractionated PBMC but with an adjusted number of NK cells. These normal values of NK-to-K562 ratios at 16:1, 8:1, 4:1 and 2:1

Table 4 Information on the four patients with expected low NK cell cytotoxic function

Patient	Clinical information
1	Epstein–Barr virus-induced secondary HLH without a discovered genetic cause
2	Epstein–Barr virus-induced secondary HLH without a discovered genetic cause
3	Epstein–Barr virus-induced secondary HLH without a discovered genetic cause
4	Anhidrotic ectodermal dysplasia with immune deficiency; NK cytotoxic defect previously confirmed in year 2006

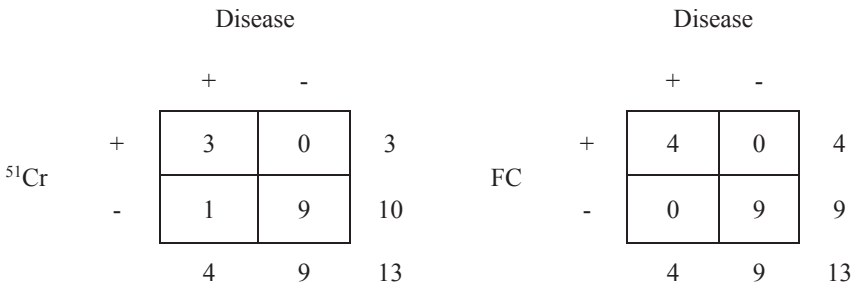

Fig. 4 Comparison of diagnostic sensitivity and specificity between flow cytometry and the ^{51}Cr-release assay.

were calculated from 40 healthy adult individuals. Understandably, the sample size used to generate of our normal value could increase to enhance representation of the local Hong Kong population. After performing sample size estimation using our 40 normal values, we projected a statistically representative sample size to be approximately 350 (confidence level 95%). Hence testing of normal controls to further expand our sample pool is ongoing in our laboratory, and the normal values will be revised once our sample size reaches the target. Interestingly, it is also theoretically possible to use unadjusted PBMC in determining cytotoxicity to specific NK cell-to-target ratios via the help of mathematical modelling. By fitting our FC assay data into mathematical functions, such as the non-linear least squares technique, calculation of cytotoxicity across the entire range of effector-to-target ratios (e.g., LD_{25} and maximal cytotoxicity values) should be feasible.[25] However, we have not evaluated how well our data could be combined with these algorithms and could be using these altogether clinically, as it is beyond the scope of this report. Nevertheless, incorporation of mathematical models is definitely worth exploring in future, since this should further simplify workflow of our FC assay by not needing adjustment of PBMC number.

Despite many reports published in the literature on measuring NK cell cytotoxicity by FC, the method is not standardised across laboratories and practices can differ significantly. Such differences include variations in the effector-to-target ratios, the use of rIL-2 to augment the NK response, the use of other fluorochromes (e.g., CFSE or BCECF-AM, etc.) to label the target K562 cells, complete NK cells purification by magnetic beads, etc.[16–20] Our method mainly differs from these earlier reports in two ways: (1) we utilised DiO as our K562 labelling dye, and (2) our effector-to-target ratio is unique with NK cell number adjusted amongst the unfractionated PBMC. We chose to use DiO because it was economical and easy-to-handle. DiO also has good labelling efficiency and it was noted in literature to display less leakage in comparison to other labelling dyes.[20–22] In fact, we have compared the effect of DiO with CFSE, another commonly used labelling dye for other FC-based NK function assays, and we found significant CFSE leakage over the course of the assay that had affected the differentiation of the K562 cells from the PBMC (Supplementary Fig. 1, Appendix A), which ultimately compromised the assay's performance. In terms of the effector cells, we decided to work with unfractionated PBMC for two main reasons. Firstly, NK cell purification would induce cell loss in the process, and thus a greater volume of specimen would be required from the test subjects, making it less ideal for paediatric patients. Furthermore, purification procedures would add further processing time, making the workflow too long to be practical in a clinical setting. Nevertheless, we investigated whether purified NK cells would impact on the results from unfractionated PBMC and we found no significant difference (data not shown). Moreover, using a positive selection method to deplete NK cells, we titrated different concentrations of bystander cells and confirmed that these cells do not induce cytotoxicity in our assay (Supplementary Fig. 2, Appendix A). This was expected because the other cells that can elicit cytotoxicity are T-cells but these cells are MHC-restricted, and therefore they should react minimally with the K562 targets. One thing worth mentioning is that in our FC assay, while NK cell numbers were adjusted based on the NK cell percentage obtained from lymphocyte subset, the adjustment of NK cell numbers may not be exact because the PBMC mixture would also contain monocytes that were not accounted for by our lymphocyte subset results. However, we assumed this interference by monocytes would be negligible because in our experience the percentage of monocytes after PBMC isolation is usually less than 10–12%. Another limitation of our FC assay is that our reference values are generated by adult samples that may not be physiologically relevant to paediatric specimens. Unfortunately, determination of paediatric reference values is a real challenge due to scarcity of such samples. One way to overcome this is to use several paediatric samples to validate our existing adult range. This way, not as many paediatric volunteers would be needed as those normally required for establishing a new reference range.

NK cell activity can also be evaluated by measuring intracellular levels of perforin and granzymes, or by assessing surface staining of the lysosomal-associated membrane protein 1 (CD107a) which correlates cytolytic granules degranulation.[26,27] These parameters can be detected by FC and are recognised as important markers in HLH, with increasing supporting evidence that reduced expression of perforin and CD107a should be included as part of diagnostic criteria for primary HLH.[28,29] Determining defects in component(s) that are involved in activation, docking or priming of the lytic granules also allows classification of the congenital forms of HLH that are known as familial haemophagocytic lymphohistiocytosis (FHL). For instance, FHL2 is caused by perforin deficiency in which the granule release mechanism is not affected. Interestingly, a recent publication by Rubin *et al.* suggested perforin and degranulation assays by FC should be preferentially performed because they found higher sensitivity in these assays over the ^{51}Cr-release assay for screening patients for primary HLH diseases.[30] However, whether these assays have better sensitivity in detecting HLH than our FC-based NK cytotoxicity assay requires further evaluation.

Nevertheless, it is recommended that a CD107a screening and perforin expression analysis should be performed in all patients suspected of HLH, followed by measurement of NK cell cytotoxicity as a second line test if perforin or NK degranulation is found to be abnormal. Therefore, our FC-based assay could supplement the perforin intracellular staining and degranulation assays for the diagnosis of HLH. Indeed, it is reported that ^{51}Cr-release assay has excellent negative predictive value and would be helpful as a confirmatory test to rule out certain forms of HLH.[29] It is also worth noting that the NK cytotoxicity assay measures the overall cytotoxic potential of the NK cells, whereas the perforin intracellular staining and degranulation assays only provide dissection of a particular cytotoxic mechanism. Hence, the NK cytotoxicity assay as a follow-up test may be useful to reveal defects in other parts of the NK cell cytotoxic pathway.

In summary, we have demonstrated the applicability of FC in measuring NK cell function in the clinical laboratory. This assay has many advantages, such as eliminating the use of radioactive chemicals, and the test allows analysis of NK cell function at a single-cell level. The turnaround time of FC is rapid, able to be completed in approximately 6 hours which is within an 8-hour work shift typical in a clinical laboratory. As our sample size of healthy adults for normal values continues to increase and become more representative, the FC method should become very useful as part of a regular service in an immunopathology laboratory.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2018.12.417.

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References

1. Parvaneh N, Casanova J-L, Notarangelo LD, *et al*. Primary immunodeficiencies: a rapidly evolving story. *J Allergy Clin Immunol* 2013; 131: 314–23.
2. Rosado FGN, Kim AS. Hemophagocytic lymphohistiocytosis: an update on diagnosis and pathogenesis. *Am J Clin Pathol* 2013; 139: 713–27.
3. Usmani GN, Woda BA, Newburger PE. Advances in understanding the pathogenesis of HLH. *Br J Haematol* 2013; 161: 609–22.
4. McCusker C, Warrington R. Primary immunodeficiency. *Allergy Asthma Clin Immunol* 2011; 7 (Suppl 1): S11.
5. Borte S, von Döbeln U, Hammarström L. Guidelines for newborn screening of primary immunodeficiency diseases. *Curr Opin Hematol* 2013; 20: 48–54.
6. Bonilla FA, Bernstein IL, Khan DA, *et al*. Practice parameter for the diagnosis and management of primary immunodeficiency. *Ann Allergy Asthma Immunol* 2005; 94 (Suppl 1): S1–63.
7. Filipovich AH. Hemophagocytic lymphohistiocytosis (HLH) and related disorders. *Hematol Am Soc Hematol Educ Program* 2009: 127–31.
8. Pachlopnik Schmid J, Schmid JP, Côte M, *et al*. Inherited defects in lymphocyte cytotoxic activity. *Immunol Rev* 2010; 235: 10–23.
9. Risma K, Jordan MB. Hemophagocytic lymphohistiocytosis: updates and evolving concepts. *Curr Opin Pediatr* 2012; 24: 9–15.
10. Egeler RM, Shapiro R, Loechelt B, *et al*. Characteristic immune abnormalities in hemophagocytic lymphohistiocytosis. *J Pediatr Hematol Oncol* 1996; 18: 340–5.
11. Cao L-F, Krymskaya L, Tran V, *et al*. Development and application of a multiplexable flow cytometry-based assay to quantify cell-mediated cytolysis. *Cytom J Int Soc Anal Cytol* 2010; 77: 534–45.
12. Thakur A, Zaman A, Hummel J, *et al*. Single-colour flow cytometric assay to determine NK cell-mediated cytotoxicity and viability against non-adherent human tumor cells. *Biotechnol Lett* 2011; 34: 447–53.
13. Papadopoulos NG, Dedoussis GV, Spanakos G, *et al*. An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. *J Immunol Methods* 1994; 177: 101–11.
14. Zaritskaya L, Shurin MR, Sayers TJ, *et al*. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines* 2010; 9: 601–16.
15. Klöß S, Bochennek K, Huenecke S, *et al*. A novel five-colour flow cytometric assay to determine NK cell cytotoxicity against neuroblastoma and other adherent tumour cells. *J Immunol Methods* 2007; 325: 140–7.
16. Burkett MW, Shafer-Weaver KA, Strobl S, *et al*. A novel flow cytometric assay for evaluating cell-mediated cytotoxicity. *J Immunother* 2005; 28: 396–402.
17. Kasatori N, Ishikawa F, Ueyama M, *et al*. A differential assay of NK-cell-mediated cytotoxicity in K562 cells revealing three sequential membrane impairment steps using three-color flow-cytometry. *J Immunol Methods* 2005; 307: 41–53.
18. Mhatre S, Madkaikar M, Ghosh K, *et al*. Rapid flow cytometry based cytotoxicity assay for evaluation of NK cell function. *Indian J Exp Biol* 2014; 52: 983–8.
19. Piriou L, Chilmonczyk S, Genetet N, *et al*. Design of a flow cytometric assay for the determination of natural killer and cytotoxic T-lymphocyte activity in human and in different animal species. *Cytometry* 2000; 41: 289–97.
20. Kane KL, Ashton FA, Schmitz JL, *et al*. Determination of natural killer cell function by flow cytometry. *Clin Diagn Lab Immunol* 1996; 3: 295–300.
21. Valiathan R, Lewis JE, Melillo AB, *et al*. Evaluation of a flow cytometry-based assay for natural killer cell activity in clinical settings: flow cytometry-based assay for NK cell activity. *Scand J Immunol* 2012; 75: 455–62.
22. Chang L, Gusewitch GA, Chritton DB, *et al*. Rapid flow cytometric assay for the assessment of natural killer cell activity. *J Immunol Methods* 1993; 166: 45–54.
23. Hatam L, Schuval S, Bonagura VR. Flow cytometric analysis of natural killer cell function as a clinical assay. *Cytometry* 1994; 16: 59–68.
24. Kim G, Donnenberg V, Donnenberg A, *et al*. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: comparisons to a 4 h 51Cr-release assay. *J Immunol Methods* 2007; 325: 51–66.
25. Karimi MA, Lee E, Bachmann MH, *et al*. Measuring cytotoxicity by bioluminescence imaging outperforms the standard chromium-51 release assay. *PLoS One* 2014; 9: e89357.
26. Ferrante A, Kiroff GK, Drew PA. Elevated natural killer (NK) cytotoxicity of mononuclear leucocytes from splenectomized patients: increase in Leu-7+ and Leu-11+ leucocytes. *Clin Exp Immunol* 1986; 64: 173–80.
27. Shabrish S, Gupta M, Madkaikar M. A modified NK cell degranulation assay applicable for routine evaluation of NK cell function. *J Immunol Res* 2016; 2016: 3769590.
28. Li Q. NK cell assays in immunotoxicity testing. In: Dietert RR, editor. *Immunotoxicity Testing*. Totowa, NJ: Humana Press; 2010; 207–19. http://link.springer.com/10.1007/978-1-60761-401-2_15
29. Hines MR, Nichols KE. Go with the flow: perforin and CD107a in HLH. *Blood* 2017; 129: 2954–5.
30. Rubin TS, Zhang K, Gifford C, *et al*. Perforin and CD107a testing is superior to NK cell function testing for screening patients for genetic HLH. *Blood* 2017; 129: 2993–9.