

## ORIGINAL ARTICLE

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# The Correlation between the Percent of CD3<sup>-</sup> CD56<sup>+</sup> Cells and NK Precursor Function

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### ABSTRACT

The number and function of human natural killer (NK) cells are generally assessed to monitor the baseline of immune function, the effect of treatment, the progress of malignancy or metastases and diseases. NK cells recognise and kill target cells in the absence of prior sensitisation and are able to defend the host from infection or prevent the progression of a disease. Human NK cells express CD16 and CD56 which are (massively) being used as a major hallmark for the NK cell. The purpose of this study was to identify the unique subsets of peripheral blood mononuclear cells (PBMC) (%CD3<sup>-</sup>CD56<sup>+</sup> cells) by flow cytometry and to determine whether there is any correlation with functionally mature progeny of (NKp) precursor after five days of culture.

The correlation was analysed using samples obtained from 120 Caucasian patients. 20-30ml of whole blood was collected in sterile tube containing preservative free sodium heparin and a similar sample was obtained after five days. Maturation of NKp required the continuous presence of recombinant interleukin 2 (rIL-2), or interleukin 15 (rIL-15) and functional maturity of NK cells was determined by their ability to lyse target cells from the K562 cell line.

The NK precursor frequency was measured by limiting dilution analysis (LDA), which The NKpf assay was set up with a range of cell dilutions from 40,000 to 625 per 100µl/well in 96 well culture plates. At the end of the culture period the K562 cell line labelled with Europium (Eu-K562) was added and Eu release measured in culture supernatants using time-resolved fluorometry. The PBMC were set up in parallel cultures under various conditions. On day five cells were collected from culture plates and adjusted to 1x10<sup>6</sup> cells/ml and then mixed. The mixture was incubated and anti CD3 and anti CD56 were added. NK cells were enumerated in 120 patients by double staining with a combination of anti-CD3<sup>-</sup> and anti-CD56<sup>+</sup>.

The results of these Immunophenotyping studies by flow cytometry showed no correlation between the NKpf (natural killer precursor frequency) and the percent of CD3<sup>-</sup>CD56<sup>+</sup> cells expressed after five days confirming that CD56 was inadequate as a unique marker for functional NK cells.

**Key words:** CD3<sup>-</sup>CD56<sup>+</sup> cell; Flow cytometry; Limiting dilution analysis; Natural killer cell precursor; rIL-2; rIL-15;

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## INTRODUCTION

Maturation pathways of human NK cells are complex and poorly understood. T-cells and NK cells are both derived from common haemopoietic stem cells, and NK cells are thought to develop primarily but not exclusively along non-thymic pathways. NK cell function is an important indicator for evaluating the baseline of human immune defence, monitoring the progress or progenies during the cancer immunotherapy or in the treatment of AIDS. The frequency of natural killer cell precursors (NKpf) that was measured in human peripheral blood mononuclear cell (PBMC) samples where functional maturity was reflected in lysis of K562 target cells.<sup>6,28</sup> Different methods are being used to assess NK cell cytotoxicity such as <sup>51</sup>Cr release assay, lactate dehydrogenase (LDH) release assay, Eu-release assay, Calcein cytotoxicity assay, flow cytometric assay of intracellular cytokine in NK cells, MTT assay and <sup>125</sup>IUDR-release assay or enumeration NK cell population by flow cytometry.<sup>3,4,9,14,15,17,22</sup> NK cells are effector cells in the innate immune response and are important during the early stage of infection acting without any need for prior immunization. In-vitro studies have indicated that NK cells play a vital role in defence against bacterial infections, virus-infected cells, and malignant cells.<sup>5,13,27</sup> Patients with reduced, non-functional or absent NK cells have a high frequency of infections and cancer.<sup>2,16</sup> The rapid response of NK cells is well known as shown in normal mice that are infected with lymphocytic choriomeningitis virus (LCMV), which NK cell activity peaks on day three following LCMV infection, whereas, CD8+ T-cell activity peaks on day seven.<sup>21</sup> NK cells are also involved in host defence against leukaemia.<sup>20</sup>

Normal human NK cells do not express CD3; however, they express the interleukin 2 receptor (IL-2R), CD16 and CD56. The latest cell marker is considered as being a major hallmark, it is also expressed on some other mature haematopoietic cells, including NKT-cells, adult neural and muscular tissues, as well as embryonic tissues.<sup>1,11</sup> CD16 is expressed on macrophages, mast cells, and neutrophils.

Activated T-cells release IL-2, which in turn causes NK and T-cell activation, proliferation and cytokine induction. IL-2 also stimulates a number of other cells.<sup>8,19,23</sup> IL-15 is produced by bone marrow stromal cells, dendritic cells as well as keratinocytes, and shares

many of the biological properties of IL-2. Signals transduced through IL-15R cause NK cells to produce IFN $\gamma$ , GM-CSF and TNF $\alpha$ .<sup>12,24,25</sup>

The purpose of this study was to evaluate if a specific subset NK cell characterized by CD56 positivity and CD3 negativity with flow cytometry correlated with functionally active NK cell after five days of culture or not. We also investigated the correlation between the percentage of CD3<sup>+</sup>CD56<sup>+</sup> cells and the frequency of NK precursors (NKpf).

## MATERIALS AND METHODS

### Subjects Studied

The experiments described in this study were initially approved by the local hospital research ethics committee and an informed consent was obtained from all volunteers. The correlation between the CD3<sup>+</sup>CD56<sup>+</sup> cell population and NKp cytotoxic function was analyzed using samples obtained from 120 Caucasian patients. They were between 40 and 87 years of age and all were awaiting surgery for osteoarthritis. Patients were excluded from this study if they had a pre-existing infection, a previous blood transfusion, malignancy, autoimmune disorders or diabetes. To avoid individual variation during the experiment, venous blood was taken from one healthy male adult (AG) at different times. PBMC were isolated and then stored in liquid nitrogen until the testing date.

### Collection and Isolation of Peripheral Blood Mononuclear Cells (PBMC)

20-30 ml of whole blood was collected in a sterile tube containing preservative-free sodium heparin (25 units/ml of blood) pre-operation. A similar sample was obtained 5 days post-operation.<sup>10</sup>

### Complete Culture Media (CCM)

The complete culture medium (CCM) consisted of RPMI 1640 (Sigma) supplemented with 10% heat inactivated human AB serum from normal healthy male donors (Sigma) and 3mM L-glutamine (Sigma). Antibiotics were not added to CCM to reduced assay sensitivity.

### Addition of rIL-2 and rIL-15

rIL-2 (Eurocetus, Amsterdam) diluted in CCM was added to cultures in a final concentration of 25 Cetus unit/ml. The rIL-15 (R&D, UK) was similarly diluted

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to a final concentration of 10 ng/mL. Both rIL-2 and rIL-15 were added on day 0 when setting up the assay.

### Quantitation of NK Precursor Frequency (NKpf) with PBMC

The cytotoxicity of NKp was measured by europium (Eu) release method based on the limiting dilution analysis as previously described (10). Briefly, to set up NKpf assay, PBMC were thawed and titrated in seven two-fold dilutions in CCM starting with a concentration of  $4 \times 10^4$  and finishing with  $0.0625 \times 10^4$  PBMC per 100  $\mu$ l/well in a Falcon, 3077 96-well round-bottomed culture plates (i.e.  $4 \times 10^4$ ,  $2 \times 10^4$ ,  $1 \times 10^4$ ,  $0.5 \times 10^4$ ,  $0.25 \times 10^4$ ,  $0.125 \times 10^4$ ,  $0.0625 \times 10^4$ ) using 24 replicates per dilution. The baseline control consisted of 100  $\mu$ l of CCM medium alone without PBMC. The assay plates were incubated for 5 days at 37°C in 5% CO<sub>2</sub> in a humidified atmosphere. Functional maturity of NK cells was determined by their ability to lyse target cells from the K562 cell line. The NK precursor frequency was measured by dilution analysis, which at the end of the culture period the K562 cell line labelled with Europium (Eu-K562) was added and Eu-release

was measured in culture supernatants using time-resolved fluorometry.<sup>10</sup>

### Immunophenotyping Studies

The PBMC were set up in parallel cultures under various conditions as mentioned above. On day 5 the cells were collected from culture plates and adjusted to  $1 \times 10^6$  cells per ml in RPMI 1640. 5  $\mu$ l (an equivalent of 1  $\mu$ g) of each antibody was added to 100  $\mu$ l of cell suspension with  $1 \times 10^5$  cells/ml and then mixed. The cell/antibody mixture was incubated at 4°C for 30 minutes, washed once and spun at 700g for 10 minutes, and then resuspended in 300  $\mu$ l of RPMI 1640. Finally the flow cytometric analysis was performed using Coulter Epics®XL (Luton, UK). Monoclonal antibodies specific for the human cell differentiation (CD) marker were used for two-colour flow cytometric analysis of CD3<sup>+</sup>CD56<sup>+</sup>, and CD3<sup>+</sup>CD16<sup>+</sup> cells. The monoclonal antibodies were directly conjugated to fluorochromes and used with controls including CD56 fluorescein isothiocyanate (FITC) (Becton Dickinson, UK), CD16 FITC (DAKO), CD3<sup>+</sup> phycoerythrin (PE) (DAKO) and FITC negative (DAKO) (Figure 1).

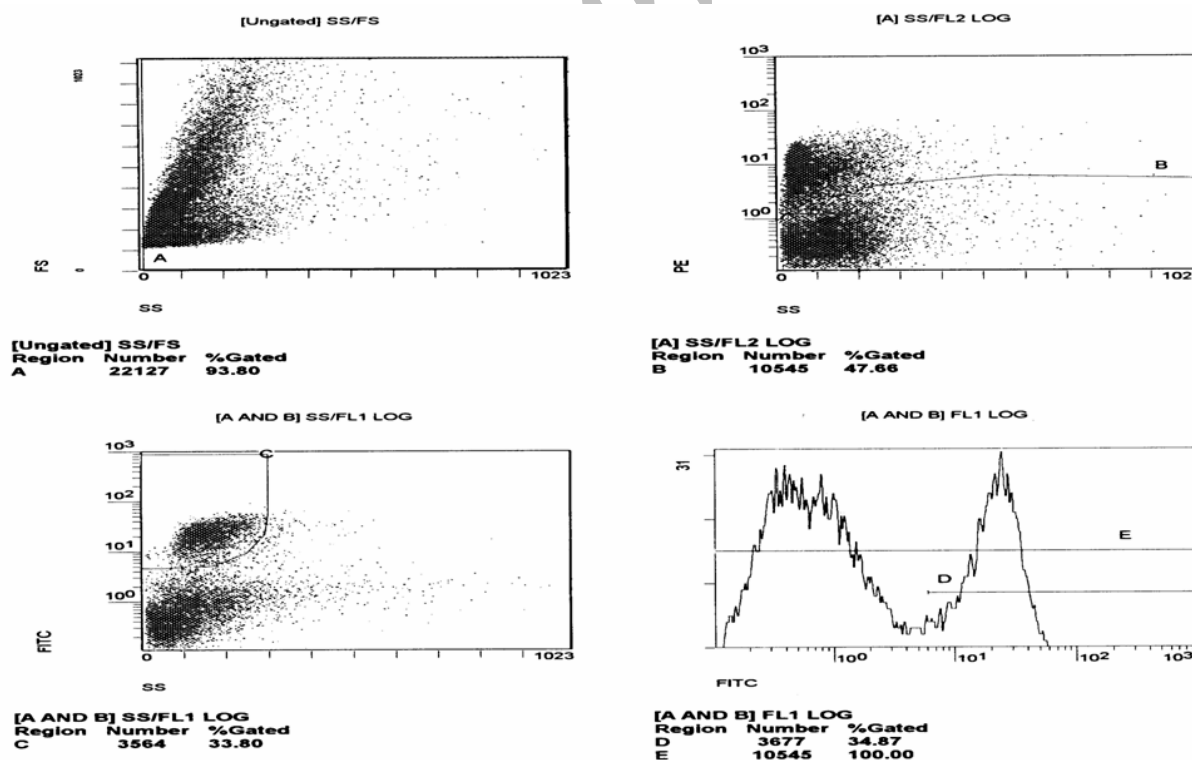


Figure 1. Schematic diagram of different gates using for enumeration of CD3<sup>+</sup>CD56<sup>+</sup> cells. Flow cytometry dot plots gated on CD3<sup>+</sup> cells (B) show populations defined by reactivity with CD56 MAb (C). Upper left quadrant forward versus side scatter; upper right, CD3<sup>+</sup> dot-plot of CD56 against side scatter, lower right is a histogram of CD56 expression in CD3<sup>+</sup> population.

Cells of interest were defined as those expressing CD56 or CD16 as a percentage of the total CD3 negative cell population by flowcytometric analysis. These were selected using electronic gating with double staining by combined antibodies against the CD3 and CD56 or combining CD3 and CD16. The CD3<sup>-</sup> cells were selected by drawing the region (B) horizontally against the region (A) in gate-2; only those cells in the region (B) were analysed in the next step. After the identification of CD56<sup>+</sup> or CD16<sup>+</sup> cells in the CD3<sup>-</sup> population, these cells were selected in gates3 by drawing region (C) vertically against the gate-3 in CD3<sup>-</sup> population. A histogram of CD56 or CD16 expression was shown in region (D) in gate4. Cells in region (C) or (D) were analysed as the percentage of CD56 positive cells in the CD3<sup>-</sup> cell population.

## RESULTS

### Effect of IL-2 and IL-15 on the Percentage of CD3<sup>-</sup> CD56<sup>+</sup> Cells

Figure 2 shows the mean percentage of the total CD3<sup>-</sup> population expressing CD56 and CD16 after five days of culture with different concentrations and

combinations of rIL-2 and rIL-15 which were added on day zero, and a control containing no cytokines added on day zero or on day five. The significance of the difference in the percentage of CD3<sup>-</sup>CD16<sup>+</sup> and CD3<sup>-</sup>CD56<sup>+</sup> cells in relation to NKpf was analyzed by the two-tailed student 't' test. The results obtained when no cytokines were added showed that there was neither any changes in the percentage of CD3<sup>-</sup>CD16<sup>+</sup> and CD3<sup>-</sup>CD56<sup>+</sup> cells on day zero nor on day five. However, after adding rIL-2 or rIL-15, a significant boost in the percentage of CD3<sup>-</sup>CD56<sup>+</sup> cells was observed (p<0.05).

These results were obtained from the healthy adult (AG) sample, while venous blood was taken at different times. The results obtained with no cytokines showed that there was no change in the percentage of CD3<sup>-</sup>CD56<sup>+</sup> on day zero and day five, while after adding cytokines, rIL-2 or rIL-15, a significant boost (p<0.05) in the percentage CD3<sup>-</sup>CD56<sup>+</sup> cells was observed. The results obtained from five experiments showed a significant increment in the percent of CD3<sup>-</sup>CD56<sup>+</sup> cells between days two and five suggesting that NK cell differentiation is cytokine sensitive and time dependent.

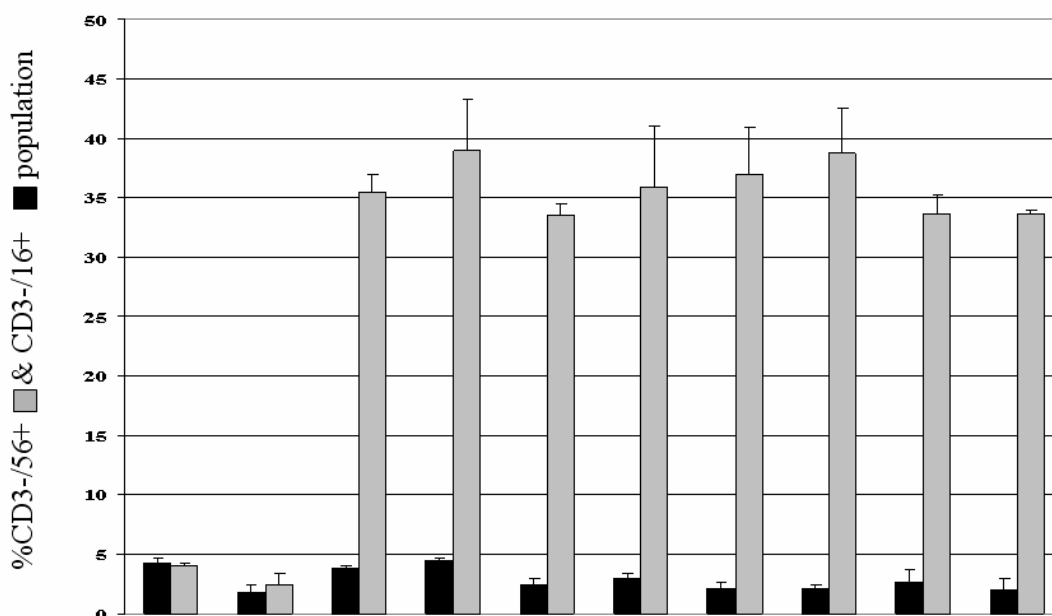


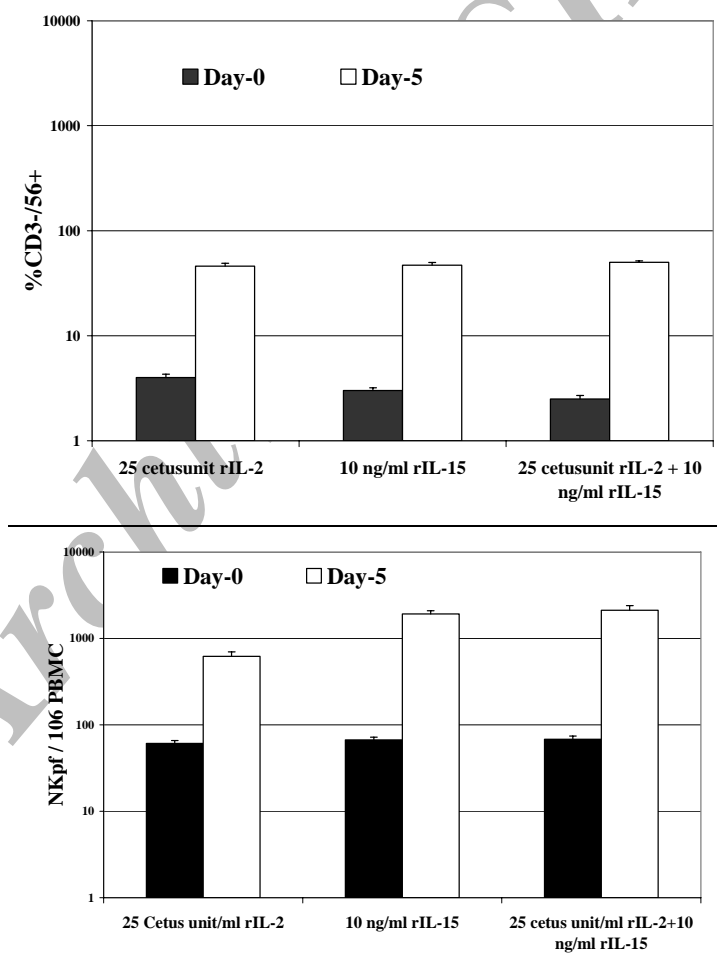
Figure 2. Mean percentage (95% CI) of total CD3<sup>-</sup> population expressing the CD3<sup>-</sup>CD56<sup>+</sup> and CD3<sup>-</sup>CD16<sup>+</sup> phenotypes after five days culture with no cytokine or with different combinations of rIL-2 and rIL-5 added on day zero. Results are the mean of five experiments with cell from AG.

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### Effect of rIL-2 and rIL-15 on the Percentage of CD3<sup>+</sup>CD56<sup>+</sup> Cells and NKp

The percentage of CD3<sup>+</sup>CD56<sup>+</sup> and NKp cells were tested after 0 and 5 days of incubation. The rationale was that previous studies stated that the differentiation and maintenance of NKp cells in culture was dependent on the continuous presence of cytokines. The majority of potentially functional NK cells were reflected on day five and there was also a greater sensitivity during the clonal expansion phase.<sup>10</sup> The results obtained from five experiments showed a significant increase in the percent age of CD3<sup>+</sup>CD56<sup>+</sup> cells between days zero and five suggesting that NK cell differentiation was cytokine sensitive and time dependent, which is consistent with the observed increase in functional NK cells (Figure 3a). The CD3<sup>+</sup>CD16<sup>+</sup> population was higher in assays cultured with rIL-2 as compared to the

assays cultured with rIL-15. Monocytes are also CD3<sup>+</sup>CD16<sup>+</sup>, and it is possible that the large increase seen in CD3<sup>+</sup>CD16<sup>+</sup> population in culture containing rIL-2 as compared to rIL-15 was due to rIL-2 driven proliferation of monocytes as well as NK cells. The CD3<sup>+</sup>CD56<sup>+</sup> population increased both in the presence of rIL-2 and rIL-15, but the last appeared to be associated with a higher increase than the former one (Figure 3a). In the next attempt the effects of rIL-2 and rIL-15 were tested both separately and together. These observations were confirmed in further three experiments. In all Immunophenotyping studies the percentage of CD3<sup>+</sup>CD56<sup>+</sup> cells were the same using different combinations and concentrations of rIL-2 and rIL-15, whereas the NKp obtained with IL-15 was significantly higher than the one obtained with rIL-2 (Figure 3b).



**Figure 3. a)** Mean percentage of total CD3<sup>+</sup> population at days zero and five expressing CD3<sup>+</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD16<sup>+</sup> (95% CI) on a log<sub>10</sub> scale ranging from 1-104 with different combinations of rIL-2 and rIL-5 added on day zero. The values shown are the mean of four experiments with cells from AG. **b)** Mean NKp estimates (95% CI) on a log<sub>10</sub> scale ranging from 1-104 at days zero and five with different combinations of rIL-2 and rIL-5 added on day zero. The values shown are the mean of five experiments with cells from AG.

Mean percentage of total CD3<sup>+</sup> population at days zero and five expressing CD3<sup>+</sup>CD56<sup>+</sup> and CD3<sup>+</sup>CD16<sup>+</sup> (95% CI) on a log<sub>10</sub> scale ranging from 1-10<sup>4</sup> with different combinations of rIL-2 and rIL-5 added on day zero. The values shown are the mean of four experiments with cells from AG. b) Mean NKpf estimates (95% CI) on a log<sub>10</sub> scale ranging from 1-10<sup>4</sup> at days zero and five with different combinations of rIL-2 and rIL-5 added on day zero. The values shown are the mean of five experiments with cells from AG.

### Percentage of CD3<sup>+</sup>CD56<sup>+</sup> Cells and NKpf Estimates in Patients

The next step was to determine whether unique subsets of PBMC could be identified by flowcytometry that correlated with functionally mature progeny of NKp after five days of culture, and to determine whether or how the percentage of CD56<sup>+</sup> cells correlated with NKpf estimates.

Patients were divided into 5 groups depending on the type of blood transfusion. Figure 4a shows the percent of CD56<sup>+</sup> cells in the CD3<sup>+</sup> population for all patients. The question was whether or not the enumeration of NK cells by flow cytometry could substitute for the assessment of NK cytotoxic function. This figure shows the percentage of the total cell population that expressed CD3<sup>+</sup>CD56<sup>+</sup> cells after five days of culture with rIL-2 and rIL-15 added on day zero. There was no difference in the mean percentage of CD3<sup>+</sup>CD56<sup>+</sup> cells in all patient groups. There was a significant decrease in NKpf estimate post-operatively in patients who were transfused with allogeneic (leukodepleted and non-leukodepleted) blood, autologous pre-deposit blood as well as non-transfused group. There was also a significant increase in NKpf estimates in those who received autologous salvaged blood (Figure 4b).

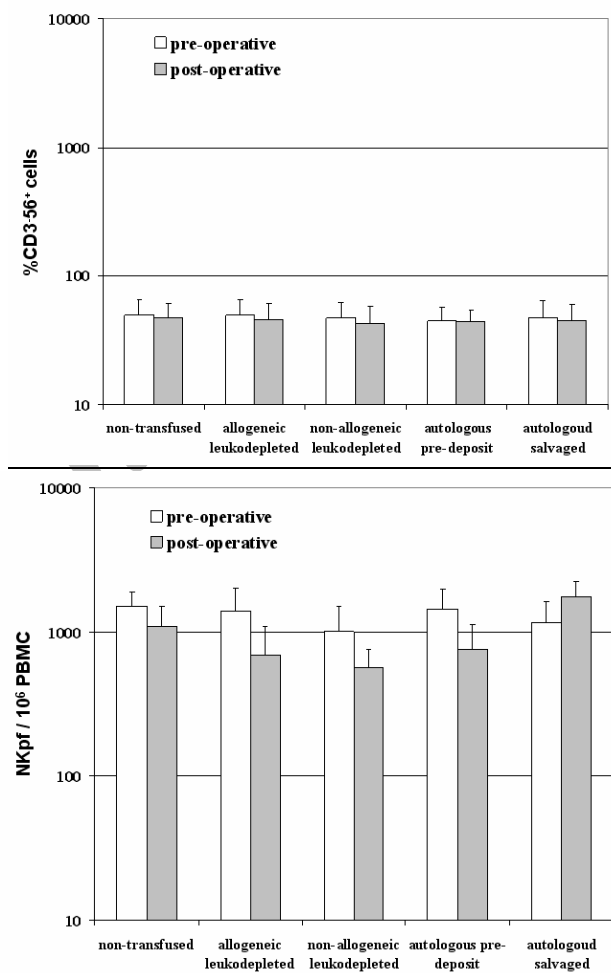


Figure 4. a) Mean percentage of total CD3<sup>+</sup>CD56<sup>+</sup> (95% CI) population on a log<sub>10</sub> scale ranging from 10-10<sup>4</sup> in five patients groups. b) Mean NKpf estimates on a log<sub>10</sub> scale ranging from 1-10<sup>4</sup> in five patients groups.

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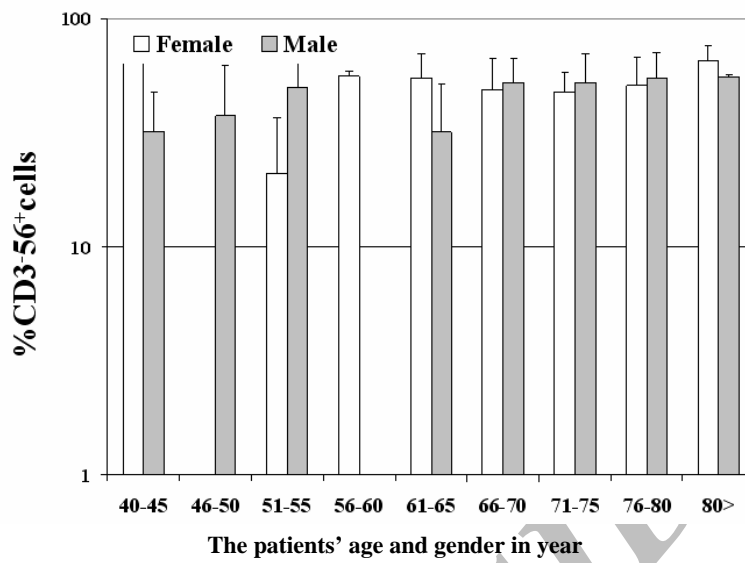


Figure 5. Mean percentage (95% CI) of total CD3 negative population that express CD56 plotted on a log<sub>10</sub> scale ranging from 1-10<sup>2</sup> distributed by age and gender.

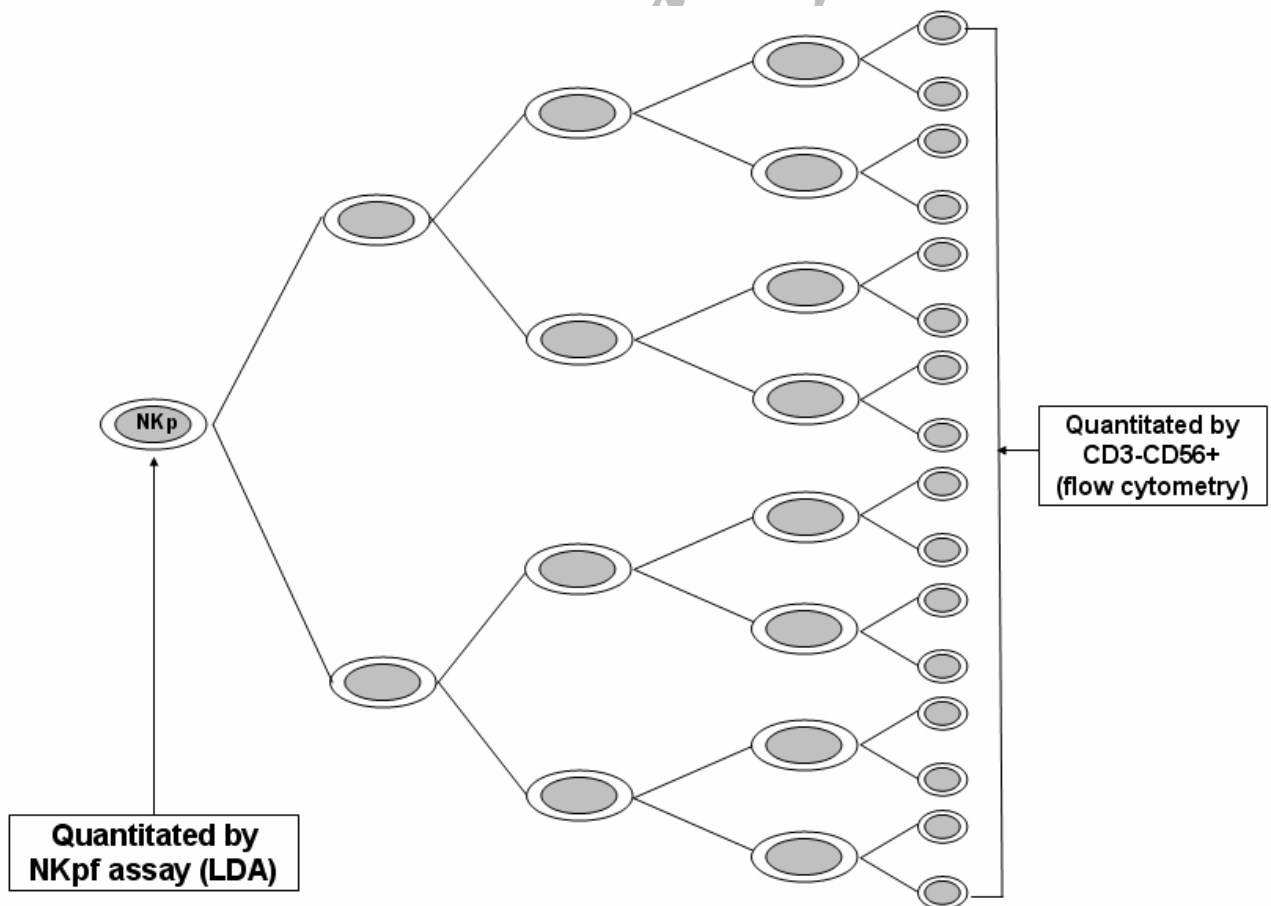


Figure 6. Schematic diagram of measurement of the frequency of NK cell precursors (NKpf) by LDA and enumeration of percent CD3-CD56<sup>+</sup> cells by flow cytometry.

As shown there was no correlation between NKpf and the percentage of CD3<sup>+</sup>CD56<sup>+</sup> cells. NKp cells may have been in the different state of cytotoxic activation against K562 target or the numbers of functionally mature NKp participating in cytotoxicity against K562 target varied among individuals.

#### CD3<sup>+</sup>CD56<sup>+</sup> Cell Population and Clinical Variables

There was a huge individual variation of percent CD3<sup>+</sup>CD56<sup>+</sup> among patients between 10-74 years of age in females and 15-83 years of age in males (Figure 5).

Figure 6 is schematic summary of the NKpf measurements by LDA and the enumeration of percent CD3-CD56+ cells by flow cytometry.

### DISCUSSION

NK cells are bone marrow derived- lymphocytes that make up to 15 percent of the circulating lymphocytes in the blood.<sup>28</sup> Considering that NK cells are the first line defence against micro organisms and malignant cells.<sup>6,28</sup> NK cell monitoring is an important indicator for evaluating the baseline of human immune defence, the progress or progenies during the cancer immunotherapy and the treatment of AIDS.

With the flow cytometry method, the number of NK cells in the circulating blood is determined by double or triple staining using the monoclonal antibodies against CD3, CD16 and CD56. For a precise enumeration of NK cells among PBMC, the sample should be stained by a combination of anti-CD3 and anti-CD56 (double staining) or including anti-CD16 to the previous Abs (triple staining).<sup>14</sup> Furthermore, it is possible to quantitate activated NK cells by flow cytometric analysis of HLA-DR, CD25 (IL-2R $\alpha$ ), CD69 and CD71 (transferrin receptor) expression in the CD3<sup>+</sup>CD56<sup>+</sup> cell population. However, it is still not definite that the expression of these activation markers correlate with NK cell cytotoxic function.<sup>26,27</sup>

Our attempts to relate cellular immunophenotype with NK cytotoxic function have also previously been made by others.<sup>18,27,28</sup> In humans CD16 exists in two isoforms: a 56-60 KDa trans-membrane isoform (Fc $\gamma$ RIIA) mainly expressed on NK cells, mast cells and monocytes/macrophages and a GPI-linked 48 KDa isoform (Fc $\gamma$ RIIB) expressed exclusively on neutrophils. CD16 may also be expressed on CD3<sup>+</sup> T-cells in certain individuals. CD56 is expressed on some

human haemopoietic cells including NK cells and a sub-population of T lymphocytes termed NKT-cells. It is also expressed in adult neural tissues, muscular and embryonic tissues. A number of tumour cell types are positive for CD56 including some of the myeloid leukaemias, myelomas, neuroblastomas, Wilms' tumour and small cell carcinomas.<sup>1,7,11</sup>

Our observations suggested that rIL-2 alone preferentially activated CD3<sup>+</sup>CD16<sup>+</sup> as compared to CD3<sup>+</sup>CD56<sup>+</sup> cells; whereas, rIL-15 activated a higher percentage of CD3<sup>+</sup>CD56<sup>+</sup> population. This result suggests that although IL-2R and IL-15R can both be ligated by IL-2 and IL-15 by virtue of the shared  $\beta$  and  $\gamma$ c chain, they reserve their highest specificity for their specific cytokines. Furthermore the result suggests these proliferation that are IL-2R+, IL-15R-; IL-2R+, IL-15R+ and IL-2R-, IL-15R+.

The lack of correlation between percent CD3<sup>+</sup>CD56<sup>+</sup> cells and NKpf is consistent with the notion that although most NK cells may express CD56<sup>+</sup>, not all cells expressing CD56<sup>+</sup> exhibit NK function, and circulating CD56<sup>+</sup> cells may vary in their state of cytotoxic activation (26). These results support the previous observations that the enumeration of NK cells by flow cytometry is unable to reliably measure NK cytotoxic function and that the CD3<sup>+</sup>CD56<sup>+</sup> cell is an inadequate marker.<sup>18,27,28</sup>

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