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Practical NK cell phenotyping and variability in healthy adults

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Abstract

Human natural killer (NK) cells display a wide array of surface and intracellular markers that indicate various states of differentiation and/or levels of effector function. These NK cell subsets exist simultaneously in peripheral blood, and may vary amongst individuals. We examined variety amongst selected NK cell receptors expressed by NK cells from normal donors, as well as the distribution of select NK cell subsets and NK cell receptor expression over time in several individual donors. Peripheral blood mononuclear cells (PBMCs) were evaluated using flow cytometry via fluorochrome-conjugated antibodies against a number of NK cell receptors. Results were analyzed for both mean fluorescence intensity (MFI) and the percent positive cells for each receptor. $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets were also considered separately, as was variation of receptor expression in NK cell subsets over time in selected individuals. Through this effort we provide ranges of NK cell surface receptor expression for a local adult population as well as provide insight into intra-individual variation.

Keywords

NK cells; immunological phenotype; primary immune deficiency; flow cytometry

1. Introduction

The detection and diagnosis of immunological diseases relies upon an ability to identify abnormalities in both the number and function of distinct populations of immune cells when compared to a reliable panel of healthy controls. The significance of maintaining a regionally relevant updated pool of age-matched normal donors for this purpose has been documented [1, 2]. Immune diseases, including Primary Immunodeficiency, typically contain quantitative and/or qualitative defects in immune cell subsets, which can include the NK cell compartment [3]. Identification of NK cell deficiencies relies on an accurate

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assessment of the frequency of $CD56^{+}$ $CD3^{-}$ lymphocytes present in peripheral blood as well as the distribution of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets [3]. In addition, unique NK cell profiles may be indicative of particular immune deficiencies, such as that caused by GATA2 mutation [4].

NK cell maturation in both mice and humans begins in the bone marrow and is thought to proceed in secondary lymphoid tissues [5], [6]. Peripheral blood in the majority is represented by mature NK cells, but also contains immature and heterogeneous NK cell subsets. $CD56^{bright}$ NK cells are thought to represent a less mature precursor of $CD56^{dim}$ NK cells [6]. In addition to representing particular stages of development, NK cell subsets have distinct functional roles. The association of these to human disease has only been minimally explored and will rely upon a robust appreciation of normal human ranges.

In addition to NK cell development, particular NK cell receptors enable specific NK cell functions. Some of these detect signatures of danger to induce NK cell function, while others identify characteristics of health to restrain NK cell activities. The topic of functionally diverse NK cell receptors has been reviewed extensively [7] [8] [9]. Those considered in the current study with regards to considering local healthy donor ranges are listed in Table 1.

While there have been several studies of extended human NK cell phenotypes, few have focused on the establishment of normative ranges and none to our knowledge has considered the extended phenotype variability over time in specific healthy individuals. Thus, we evaluated a pool of 40 adult healthy donors by multi-parametric flow cytometry using an NK cell phenotype panel in an effort to establish normative ranges. We combine this with a study of intra-individual variability in a subset of individuals in an effort to define normal acceptable alterations in NK cell receptor expression over time.

Materials and Methods

2.1 Human NK cell preparation

PBMCs were isolated from whole blood of normal donors by density centrifugation over Ficoll-Hypaque lymphocyte isolation medium (GE Healthcare Life Sciences). All human samples were obtained using written informed donor consent and were used with the approval of the Children's Hospital of Philadelphia and/or Baylor College of Medicine Institutional Review Boards for the Protection of Human Subjects. Three donors were used for the study of individual variation at intervals ranging from 1 week to 2 years.

2.2 NK cell phenotyping, flow cytometry, and analysis

Following density centrifugation, 2.5×10^5 PBMCs were washed once in FACS buffer [1% bovine serum albumin (BSA) in phosphate buffered saline (PBS)]. For biotinylated antibodies (anti-CD11b and anti-CD122) 10 μl of antibody was added to tubes and incubated for 15–20 minutes at room temperature in the dark, followed by 1 μl of streptavidin-conjugated 488 for an additional 15–20 minutes. All Staining was done in 100 μl of FACS buffer. Other surface marker antibodies or isotypic controls were then added (1:100), including anti-CD3 and anti-CD56, and incubated 15–20 minutes (Table 2). Cells were

washed twice with FACS buffer and resuspended in 300 μl FACS buffer plus 200 μl 2% paraformaldehyde. If intracellular staining was performed, 500 μl of pre-warmed CytoFix/Cytoperm (BD Biosciences) was added to tubes following surface marker staining and incubated for 15 minutes at room temperature. Cells were washed twice with Cytoperm wash buffer and specific antibody added (e.g. anti-perforin) and incubated for 15–30 minutes. Cells were washed twice with Cytoperm wash buffer. All samples were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences). PBMCs were gated based upon forward and side scatter to allow for identification of lymphocytes. Positive and negative thresholds for fluorescence signals were defined using isotype-specific negative controls. NK cells were delineated as lymphocytes that were $CD56^{+}CD3^{-}$. The fluorescence intensity of CD56 staining was used to identify $CD56^{bright}$ NK cells and, when necessary, the demarcation between $CD56^{bright}$ and $CD56^{dim}$ populations was further confirmed using CD16 on NK cells to identify the $CD56^{dim}CD16^{+}$ population. For detailed NK cell surface marker evaluation, the frequency of positive events was measured as those $CD56^{+}CD3^{-}$ cells expressing the marker of interest above that of isotype-matched negative control. All flow cytometric analyses were performed using FlowJo (Treestar, Inc.). Prism 5.0 (GraphPad Software) was used for all graphing and statistical analyses.

3. Results

To ascertain the variation in NK cell populations across a pool of healthy adult donors (n=40), a repertoire of twenty surface receptors was evaluated (Table 1). For three of these donors longitudinal data were obtained over three years. Lymphocytes were identified by FSC-SSC and NK cells were defined as $CD56^{+}CD3^{-}$ (see Supplemental Figure for gating strategy). The total number of $CD56^{+}$ $CD3^{-}$ NK cells found in the peripheral blood varied widely among normal donors as a proportion of PBMC (0.61 – 16.87%), with a mean of 6.47% (Table 3). When evaluated over time in three individual longitudinal donors total NK cells comprised a mean of 8.79±3.31, 9.54±1.96, and 5.34±2.23% of PBMCs across 3–7 longitudinal assessments (Fig 1a). $CD56^{dim}$ NK cells comprise ≥90% of peripheral blood NK cells [10, 11], and our findings are consistent (mean=90.20 ± 6.84%, range 61.68 – 98.29%). $CD56^{bright}$ NK cells generally comprise ≤10% of peripheral NK cells which was also what was identified in our cohort; mean = 8.56 ± 6.90%, range 0.63 – 38.32. Mean percentages of $CD56^{bright}$ NK cells were relatively similar amongst our three longitudinal donors but varied considerably over time within each donor (Fig. 1b). As would be expected, the same was true for the mean percentages and intra-individual variability of $CD56^{dim}$ NK cells (Fig. 1c).

3.1 Activating and accessory molecules

Activating and costimulatory molecules facilitate the cytotoxic function of mature NK cells and serve developmental functions in developing NK cells. As expected, a greater proportion of $CD56^{dim}$ NK cells expressed perforin and CD16, and they were also present at higher levels (perforin: 68.34%+, MFI 41.94; CD16: 54.38%+, MFI 85.38) than on $CD56^{bright}$ NK cells (perforin 16.15%+, MFI 15.26; CD16 26.32%+, MFI 34.53) (Table 3, Fig 2b, c). CD8α was also expressed as expected and was variable between donors (total NK cells 39.33 ± 16.75%, range 8.92 – 77.3) (Fig 2a) [12], although in our cohort $CD56^{bright}$

NK cells expressed slightly less CD8α than previously published (34%) as did the $CD56^{dim}$ subset (39%) (Fig. 2c) [13]. CD2 can serve as a costimulatory receptor on NK cells as resting NK cells can be primed by CD2 ligation with CD58 [14]. While 72% of total NK cells expressed CD2 (Fig 2a), including 69.4% of $CD56^{dim}$ NK cells and 94.1% of $CD56^{bright}$ (Fig 2c), the MFI was higher in the $CD56^{bright}$ subset (693 ± 449) than the $CD56^{dim}$ subset (228 ± 154) (Fig 2b). Adhesion molecules CD11a, CD11b, and CD18 were highly expressed both on $CD56^{bright}$ and $CD56^{dim}$ NK cells and at similar levels (Fig 2). DNAM-1, recently ascribed in murine models to have important roles in the generation of NK cell memory [15], was expressed on both $CD56^{bright}$ and $CD56^{dim}$, although the range among donors varied substantively in both subsets (Table 3, Fig. 2c).

CD94 forms heterodimers with both NKG2A (inhibitory) and NKG2C (activating) receptors and as such is more uniformly associated with the $CD56^{bright}$ population, although a subset of $CD56^{dim}$ also expresses CD94 and are thought to represent an intermediate population in the potential maturation of $CD56^{bright}$ to $CD56^{dim}$ NK cells [16]. As would be expected in our donors CD94 was present on a higher percentage of $CD56^{bright}$ (82.2±19.4) (Fig. 2c) and these also had a higher density of expression (MFI 57.1±29.0) (Fig. 2b) than those $CD56^{dim}$ NK cells that express CD94 (38.7±18.6%; MFI 15.9±10.5) (Table 3, Fig. 2). Within individual donors, CD94 expression remained relatively stable in terms of both percentage and MFI (Fig 3).

2B4 (CD244) was expressed on approximately 35% of total NK cells, with a slightly higher frequency of $CD56^{dim}$ (35.6%) than $CD56^{bright}$ NK cells (22.9%) expressing comparable densities of CD244 (Table 3, Fig. 2). The cell stress sensing activating receptor, NKG2D, was expressed on both $CD56^{bright}$ and $CD56^{dim}$ NK cells although the mean percent positive and MFI were higher on $CD56^{bright}$ (73.3%; MFI 34) than $CD56^{dim}$ NK cells (47.6; MFI 17) (Fig 2b, c). NKp30 and NKp46 are natural cytotoxicity receptors (NCR) that can promote NK cell activation and can participate in the recognition of rapidly dividing as well as certain types of virally infected cells [17]. The expression of NKp46 was diffuse although the mean percentage and MFI of NKp46 positive cells was higher on $CD56^{bright}$ (76.4%; MFI 49.4) than $CD56^{dim}$ (40.5%; MFI 20.3) NK cells. In contrast, NKp30 was expressed on approximately the same low percentage of both NK cell subsets, with very similar MFIs (Table 3, Fig 2b, c).

NK cells express the IL-2 receptor and are highly responsive to this cytokine [18]. $CD56^{dim}$ NK cells express the intermediate affinity form of the IL-2 receptor, composed of CD122 (IL-2 receptor β chain) and CD132 (common γ chain), whereas $CD56^{bright}$ can express the trimeric high affinity receptor, consisting of the intermediate form in complex with CD25 (the IL-2 receptor α chain). Therefore, $CD56^{bright}$ NK cells proliferate in response to low IL-2 concentrations (picomolar range), whereas $CD56^{dim}$ cells are less responsive *in vitro* to even higher concentrations [10]. In agreement with prior observations CD122 was expressed on both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in our cohort [10] and levels were similar on $CD56^{bright}$ and $CD56^{dim}$ NK cells (Fig 2b, c). In this study, levels of CD132 and CD25 were not tested.

3.2 Inhibitory receptors

The ligation of certain NK cell receptors by specific major histocompatibility complex (MHC) molecules inhibits the cytotoxic function of mature NK cells as a mechanism to prevent errant self-destruction or inflammation as well as allows for the "licensing" of immature NK cells so that they may be enabled for function. Many of these receptors are members of the killer immunoglobulin receptor family (KIR). Antibodies against CD158a (KIR2DL1), CD158b (KIR2DL2), and CD158e1 (KIR3DL1) were used in our study. As the KIR locus is both polymorphic and subject to allelic variability, expression would be expected to vary among donors. Furthermore, KIR expression is a signature of maturation and thus $CD56^{dim}$ NK cells express KIRs on a larger percentage of cells [7]. As expected, a higher percentage of $CD56^{dim}$ NK cells expressed KIRs when in aggregate compared to $CD56^{bright}$ NK cells (Fig 2c). There was, however, some KIR expression within the overall $CD56^{bright}$ population. Ten out of 37, 21/37, and 14/37 donors expressed CD158a, CD158b, and CD158e1, respectively, on their $CD56^{bright}$ NK cells. KIR expression was highly variable on the $CD56^{dim}$ NK cell subset. Of the three KIRs examined, CD158b was the most highly expressed on $CD56^{dim}$ NK cells however the MFI for CD158e1 was the highest of all three KIRs, demonstrating that MFI and percent positivity are unrelated with regard to KIR expression (Table 3, Fig 2d). As expected, KIR expression within individual donors over time was relatively stable (Fig 3).

3.3 Differentiation/maturity markers

NK cell maturation is staged based on the coordinated expression of cell surface markers throughout development [5]. Discrete subsets are based on the expression of multiple receptors including some of those referred to in prior sections. Owing to the experimental design of the current work, receptors here were considered individually. CD27 is a member of the TNF receptor family ligated by CD70 and is expressed on immature and $CD56^{bright}$ NK cells. While it is considered to be a fundamental marker of NK cell maturation state, it is itself not required for human NK cell development [19]. As expected, there were fewer CD27-expressing $CD56^{dim}$ NK cells (10.7%) than $CD56^{bright}$ NK cells (33.5%) (Table 3, Fig 2c). CD117 (c-kit) was expressed on at least ten times as many $CD56^{bright}$ (72.4%) as $CD56^{dim}$ NK cells (5.94%) with the MFI also higher ($CD56^{bright}$ 18.0; $CD56^{dim}$ 13.2) (Fig 2b, c). This is in agreement with prior studies and reflects the importance of this receptor in NK cell development [20] [5]).

CD57 denotes terminal maturation for $CD56^{dim}$ NK cells and is increased following cytomegalovirus infection [21], [22]. As expected, CD57 was more highly expressed on the $CD56^{dim}$ NK cell subset, particularly when considered with regards to MFI ($CD56^{bright}$ 113.6; $CD56^{dim}$ 792) (Table 3, Fig 2b). Perforin is also associated with terminal NK cell maturation and is expressed at lower levels in less mature $CD56^{bright}$ NK cells [23]. As described above, intracellular evaluations identified a small percentage of $CD56^{bright}$ (16%) but many $CD56^{dim}$ (68.3%) NK cells contain perforin at levels above background (Table 3, Fig 2c). Perforin content in individual donors over time showed a range of 45–92% in total NK cells, and was considerably higher in the $CD56^{dim}$ subset than the $CD56^{bright}$ NK cells in both percent positive and MFI (Figs 3, 4, 5).

3.4 Individual variability in NK cell receptors over time

In terms of percent positive NK cells, our three donors evaluated longitudinally had several markers in common that showed little variability over time, including CD158a, NKB1 (CD158e1), CD158b, CD8, CD11a, CD27, NKp30, CD117, CD18, and CD57 (Fig 3). In addition, several markers appear to have consistent expression amongst all three donors (CD158a, NKB1, CD158b, CD8, and CD27). The MFI of many markers was more consistent among the three donors than percent positive cells, suggesting levels of surface receptor expression are relatively conserved between subjects, whereas variability arises from the frequency of expression within the population.

Differences in the expression patterns of the receptors were also considered in the specific context of $CD56^{bright}$ and $CD56^{dim}$ subsets over time for each donor. As seen in the total NK cell population, the MFIs for the markers were less variable than the percent positive cells in both the $CD56^{bright}$ and $CD56^{dim}$ subsets for all three donors (Fig 4, 5). The KIRs (CD158a, CD158b, and NKB1) retained low overall mean MFIs in the $CD56^{bright}$ subset over time, while the $CD56^{dims}$ displayed higher MFI and less variability (Fig 4, 5). Accessory molecules, including CD2, did not vary greatly over time in the $CD56^{bright}$ subset. CD2 was highly expressed on $CD56^{bright}$ NK cells from all time points (Fig 4). CD2 was expressed over a much broader range of percent positive cells for the $CD56^{dim}$ population in all three donors, and there was more variability in CD2 expression in the $CD56^{dim}$ subset over time (Fig 5). CD11a and CD18 were expressed at very high and very consistent levels over all time points in both subsets. CD11b showed more variation, both among and within donors. CD16 expression fluctuated on the $CD56^{dim}$ subpopulation for all three donors; however, CD16 expression was also quite variable in the $CD56^{bright}$ NK cell subset over time. Donor B's $CD56^{bright}$ cells were 38% $CD16^{+}$ and did not fluctuate in the same manner as Donors A and C (Fig 4).

Expression of activation receptors CD94, NKp46, DNAM1, NKG2D, and CD244 varied over time (Fig 3). NKp30 expression on $CD56^{bright}$ NK cells was very low in all three donors with the greatest variability in Donor A (Fig 4). In the $CD56^{dim}$ subset NKp30 was quite stable and consistently expressed at low levels over time (Fig 5). CD57 showed transient low expression on $CD56^{bright}$ NK cells for each of the donors (Fig 4). In the $CD56^{dim}$ subset, CD57 was more moderately and consistently expressed in terms of percent positive cells (Donor A 29.8%; Donor B 38.1%; and Donor C 25.9%) but was slightly more variable in Donor C than in Donors A and B (Fig 5). $CD56^{dim}$ cells exhibited consistent perforin with a mean expression of 61.9, 65.6, and 59.6% for Donors A, B, and C, respectively, whereas in $CD56^{bright}$ cells it was more variable (Fig 4). The range of perforin positive $CD56^{dim}$ cells was 47.4 – 77.7% for Donor A; 51.5 – 89.3% for Donor B; and 45.5 – 74.5% for Donor C (Fig 5).

4. Discussion

Our laboratory has engaged in the discovery of NK cell abnormalities amongst heritable immune defects [2]. The assessment of NK cell receptor expression in peripheral blood provides utility by furthering our understanding of how normal NK cell receptor expression varies across a pool of normal donors and is modulated over time in a given individual. This

is an essential prerequisite to being able to understand what might be abnormal. Thus, we examined a repertoire of twenty different NK cell receptors in both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in a healthy adult population and also monitored changes in these receptors in three donors over time. By using a standardized and rigorous approach to data acquisition and analysis, we are thus able to make quantitative comparisons of NK cell receptor distribution within a large, ethnically diverse population, as well as within three healthy and immunologically normal adults.

Several immunodeficiencies present with decreased NK cell frequency, and classical NK cell deficiency is defined clinically when NK cells are <1% of PBMC [3]. Generally, NK cells comprise ~10% of lymphocytes in peripheral blood. Our data, which includes a relatively large cohort of healthy adults, reflects a wide range of NK cells within the lymphocyte compartment as reported previously [4]. The mean, however, places most donors within previously determined normal ranges. Within the same individual over time there was also variation in NK cell numbers, with each donor ranging from approximately 2–11% NK cells within PBMC over as little as seven months. This plasticity in NK cell number may relate to lifestyle and environment as many variables, including brief exercise, have been shown to affect NK cell numbers within peripheral blood [24]. This may also reflect unappreciated non-clinically apparent immune events that were contributing to altered homeostasis in the donor at the time.

A further complexity of human NK cell function is the presence of the $CD56^{bright}$ and $CD56^{dim}$ subsets, which reflect both developmental stages and discrete functional elements. As $CD56^{dim}$ NK cells make up the majority of the NK cell population in peripheral blood, the graphs depicting total NK cells are more reflective of the $CD56^{dim}$ phenotype than the $CD56^{brights}$, thus examining both subsets is important in potentially determining NK cell defects in patient populations. In addition, the relative proportion of the populations to each other is linked to known diseased states. Most notably, GATA2 mutation is associated with loss of the $CD56^{bright}$ NK cell subset [4], whereas mutation in the MCM4 gene results in a paucity of $CD56^{dim}$ NK cells [25]. Both disorders are associated with severe accompanying NK cell functional defects. Our data indicate that a wide range of $CD56^{bright}$ and $CD56^{dim}$ NK cells may be considered normal amongst individuals, or notably within the same individual over time.

For the most part, our measure of the expression of NK cell surface receptors reflected the known distinct $CD56^{bright}$ and $CD56^{dim}$ phenotypes. CD94, DNAM-1, NKG2D, and NKp46 were expressed on a higher percentage of $CD56^{bright}$ NK cells. NKp30, however, was expressed equally in $CD56^{bright}$ and $CD56^{dim}$ subsets. Overall higher expression of these activating receptors in the $CD56^{bright}$ subset was consistent over time for all three individual donors, but the levels fluctuated. 2B4 (CD244) was expressed at lower levels in $CD56^{bright}$ cells, although there was some variation both within and among donors. The lower mean percent positive expression of 2B4 in $CD56^{bright}$ cells is probably due to the fact that 6 out of 29 donors interrogated for this marker did not express any 2B4 on their $CD56^{bright}$ NK cells, while all donors expressed 2B4 on their $CD56^{dim}$ cells.

CD57 is typically considered a marker of maturity for $CD56^{dim}$ NK cells that is acquired following human cytomegalovirus (HCMV) infection, and may be a marker for HCMV memory [21], [22]. All donors expressed CD57 on their $CD56^{dim}$ NK cells. In contrast, 11/23 donors had 0% CD57+ cells within the $CD56^{bright}$ subset. As reported previously, there was a wide range of CD57 expression among donors on $CD56^{dim}$ NK cells [21]. DNAM-1 up-regulation is also associated with the acquisition of NK cell memory in mice [15], therefore the higher expression of DNAM1 on $CD56^{bright}$ in many donors is interesting given its potential association with a terminally differentiated $CD56^{dim}$ population.

Within almost all markers investigated there was a significant range amongst donors. The exception was KIRs, which individually were not expressed on as many individuals' NK cells, and CD8. This variation reflects the recent studies using mass cytometry that identified as many as 30,000 unique NK cell populations within an individual and >100,000 within the population [26]. This diversity, which is a feature of both genetics and environment, is proposed to represent multiple NK cell sub-populations [26]. The greater diversity in frequency of receptor-positive cells, as opposed to MFI, suggests that there is less variation in the surface density of molecules expressed than there is in the number of cells expressing the marker of interest. The inclusion of MFI in our study is valuable, however, as many mass cytometry studies use Boolean logic for gating, and thus assign a binary (positive/negative) label on each marker. As a number of disease states may ultimately affect levels of receptor expression [4] [27], continued focus on receptor density will likely have a valuable role.

Regarding individual variation in NK cell receptors over time, Donors B and C had a similar time frame of 7 or 8 months overall, whereas Donor A's samples spanned three years, hence these time points provide only a brief snapshot of the variation of NK cell receptor expression over time. These snapshots, however, reveal considerable variation in receptor and NK cell subset distribution. As expected, however, KIR expression remained fairly stable in all donors.

Several markers included in our panel have been linked to NK cell deficiency and as such are particularly valuable. These include the CD16 B73.1 clone, as diminished binding of this antibody accompanied by normal binding of the 3G8 clone is associated with CD16 deficiency [28]. CD27 deficiency results in NK cell functional defects and EBV viremia [19]. Perforin deficiency results in deficiency of NK cell cytotoxicity and susceptibility to hematophagocytic lymphohistiocytosis [29]. Leukocyte adhesion deficiency-I is a result of mutations in the *ITGB2* gene that encodes LFA-1, resulting in destabilized expression of CD18 and loss of LFA-1 expression. As noted previously, mutations in both *MCM4* and *GATA2* result in skewing of the $CD56^{bright}$ to $CD56^{dim}$ ratio and each are associated with NK cell dysfunction and accompanying disease. By continuing to define normal ranges for those markers associated with disease, we aim to improve the accuracy and impact of diagnoses of NK cell deficiency and related syndromes, which lead to severe and often fatal disease. It will be important in future work to define NK cell phenotypes in children to establish age-specific normal ranges. Our results presented here, however, demonstrate significant plasticity of the NK cell compartment amongst and within healthy human adults, in terms of both subset distribution and receptor expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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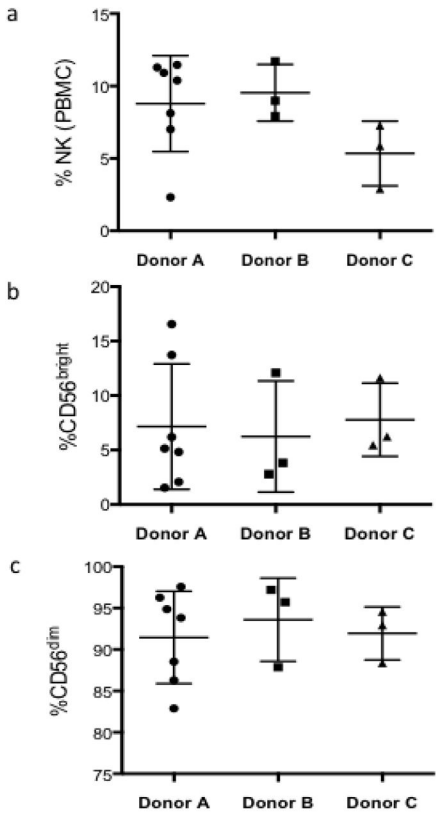

Fig. 1. Variation in NK cell frequency and subsets over time

PBMCs from healthy donor controls were analyzed by flow cytometry as described in Materials and Methods. Percent total NK cells in PBMC were defined as $CD56^{+}CD3^{-}$ (a). $CD56^{bright}$ (b) and $CD56^{dim}$ (c) subsets were defined by CD56 density and/or CD16 expression. Each data point represents one evaluation. n=7, 3, and 3 for Donors A, B, and C respectively. Mean±SD are shown.

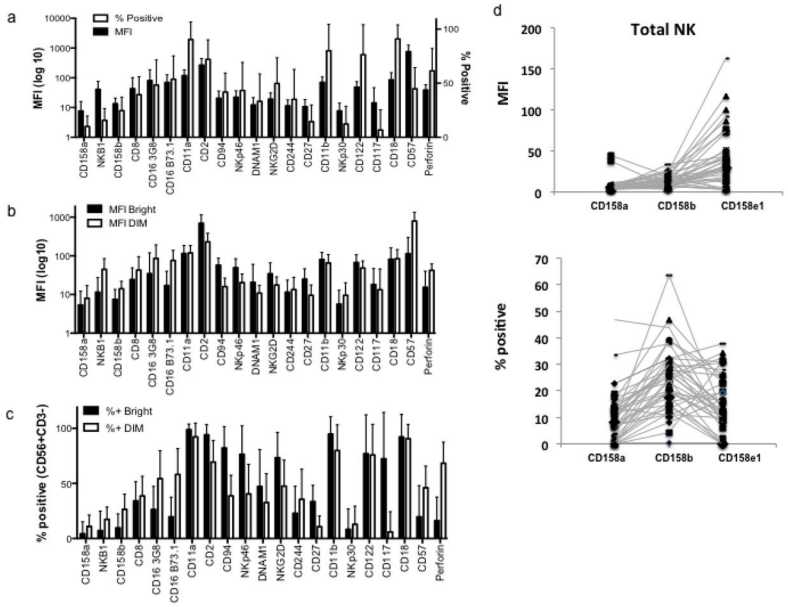

Fig. 2. Expression of markers on human NK cells derived from healthy donors

NK cells were identified as $CD56^{+}CD3^{-}$. a) Expression of markers of interest was calculated based on MFI (black, left Y axis) or percent positive above isotype control (white, right Y axis). b) MFI of markers of interest is shown for $CD56^{bright}$ (black) and $CD56^{dim}$ (white). c) % positive expression of markers of interest above isotype are shown for $CD56^{bright}$ (black) and $CD56^{dim}$ (white). n=22–40. Mean±SD are shown. d) MFI (top) or percent positive (bottom) of KIRs studied on total NK cells for each donor N=40. All bars demonstrate surface expression except for perforin, which demonstrates intracellular detection through use of a separate protocol (see *Methods*).

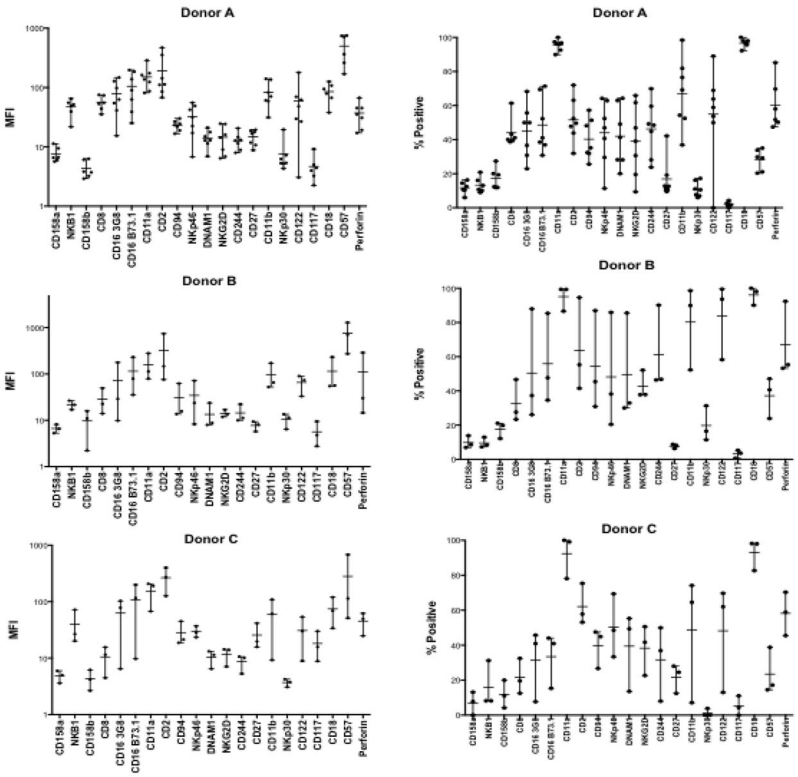

Fig. 3. Variation in NK cell marker expression in three healthy donors over time

Three individual donors were followed over time for changes in the expression of NK cell markers within the $CD56^{+}CD3^{-}$ NK cell population. Each data point represents a single evaluation (n=3–7). Expression of markers of interest is shown as MFI (left) or % positive above isotype (right). Mean±SD are shown. The longitudinal assessments cover time periods in total between 7mos and 3yrs depending upon the donor tested.

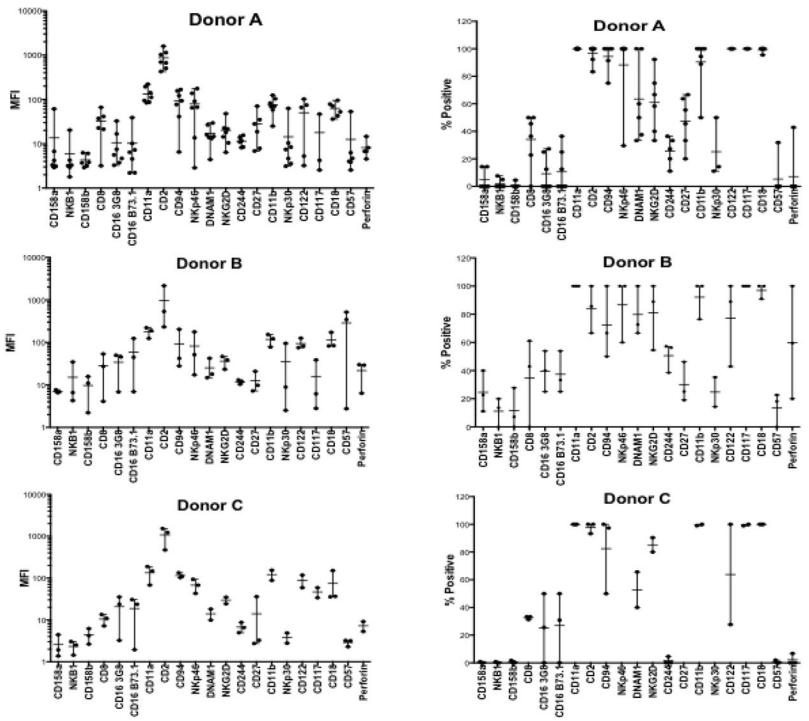

Fig. 4. Variation in NK cell marker expression on CD56^{bright} cells in healthy donors over time

CD56^{bright} NK cells were identified as in Figure 1 and markers of interest are shown as in Figure 3.

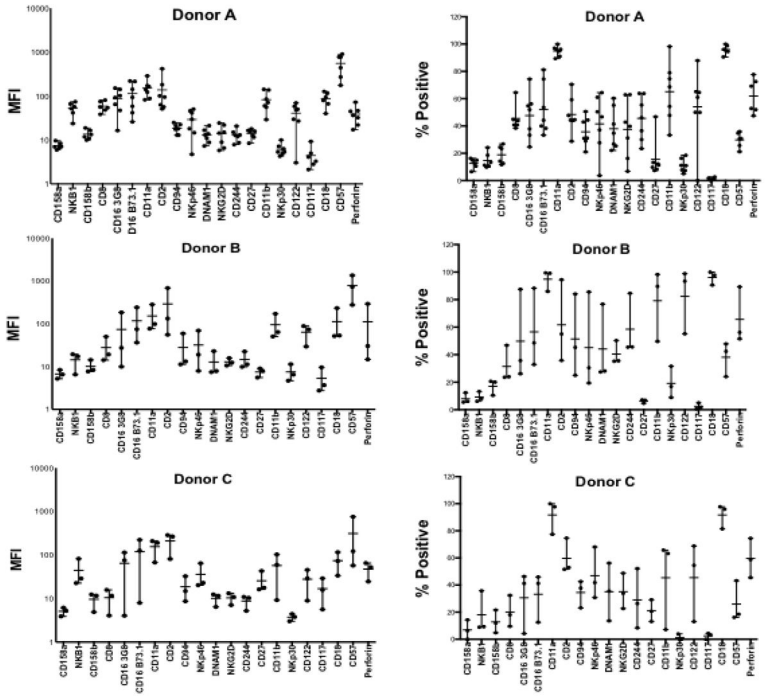

Fig. 5. Variation in marker expression on CD56^{dim} NK cells in healthy donors over time

CD56^{dim} NK cells were identified as in Figure 1 and markers of interest are shown as in Figure 3.

Table 1

NK cell receptors and functions.

Marker	Aliases	Function in NK cells	References
CD2	Sheep Red Blood Cell Receptor, Erythrocyte Receptor, Rosette Receptor, LFA-2, LFA-3 Receptor, T-cell surface antigen T11/Leu-5.	Nanotube formation in NK cells; Priming of resting NK cells; Activation of ERK 1/2 at the NK immunologic synapse.	[30], [31], [32] [14], [33]
CD8	T-lymphocyte differentiation antigen T8/Leu-2, OKT8 T cell antigen, p32, T cell co-receptor, CD8 alpha.	Prevents activation-induced apoptosis; enhances cytolytic activity; IFNγ production in HIV	[34] [12] [35] [36]
CD11a	Integrin, Alpha L (Antigen CD11a (p180), ITGAL, Lymphocyte Function Associated Antigen 1 (LFA-1), Alpha Polypeptide.	Adhesion and granule polarization; ERK 1/2 activation; co-stimulatory	[37] [33] [38] [39] [40]
CD11b	Integrin, Alpha M, Complement Receptor Type 3, Alpha subunit; CR3A;, Cell Surface Glycoprotein MAC-1 Subunit Alpha, Leukocyte adhesion receptor Mo1, Cr-3 Alpha chain, MAC1A, SLEB6.	Adhesion and degranulation.	[41] [40, 42] [43] [44, 45]
CD16	Fc Fragment of IgG, Low Affinity IIIa Receptor for (CD16a); FC-gamma RIII-Alpha 3; FCGR3A, IGFR3.	Important in NK cell development and lytic function; mediates ADCC.	[46] [47–49] [13]
CD18	Integrin beta 2, complement component 3 receptor 3 and 4 subunit, Leukocyte Cell adhesion molecule, LFA-1, MAC-1, p150,95	Essential role in target cell binding and NK cell cytotoxicity.	[39–42, 45, 50, 51] [52].
CD27	TNFRSF7 (Tumor Necrosis Factor Receptor Superfamily 7), T14, CD27L Receptor, S152, Tp55	Binds CD70; marker of immature NK; expression regulated by IL-15; immuno-regulatory	[53–55]
CD57	Beta-1,3-glucuronyltransferase 1, GLCATP, GLCUATP, Human natural killer-1 (HNK1), NK1, LEU7	Marker of differentiation, acquired after HCMV infection, potential HCMV memory marker.	[56] [57] [21] [22]
CD94	Killer Cell Lectin-Like Receptor Subfamily D, Member 1, KP43, NK cell receptor.	Pairs with NKG2A or NKG2C and binds HLA-E to form inhibitory or activating complexes, respectively. Monitors MHC-I levels.	[58] [59] [60] [61] [17]
CD117	c-kit, PBT, SCFR (stem cell factor receptor), mast cell growth factor receptor (MCGFR).	Expressed on $CD56^{bright}$ NK cell subset, suppresses apoptosis of $CD56^{bright}$ NK cells, enhances IL-2 induced proliferation and IFNγ production in $CD56^{bright}$ NKs, enhances proliferation in combination with IL-2/IL-15. Genetic disruption of c-kit and c-kit ligand in mice results in a quantitative NK cell deficiency.	[62] [63–65] [20, 66] [67, 68]
CD122	Interleukin-2 receptor beta, IL15RB, High affinity IL-2 receptor subunit beta, IL-2RB, p75, P70-75.	Common signal transducing receptor beta chain for IL-2 and IL-15. Signals through Jak1, Jak 3 and STAT5; NK cell survival.	[69] [70] [71]
CD158a	KIR2DL1/S1, NKAT1 (Natural Killer-associated transcript), MHC Class I NK Cell Receptor	KIR family of inhibitory NK cell receptors, specific for HLA-C2; inhibits NK cell cytotoxic function upon binding HLA-C2 on target cells.	[72] [7]
CD158b	KIR2DL2, NKAT6, MHC Class I NK Cell Receptor	KIR family of inhibitory NK cell receptors, specific for HLA-C1; inhibits NK cell cytotoxic function upon binding HLA-C1 on target cells.	[7, 73]
NKB1 (CD158e1)	NKB1B, KIR3DL1, NKAT-3, HLA-BW4-Specific Inhibitory NK cell receptor, AMB11	KIR family of inhibitory NK cell receptors, specific for HLA-A, HLA-B expressing Bw4 epitope; inhibits NK cell cytotoxic function upon binding HLA-A and -B8 on target cells.	[7, 72–75]
DNAM1 (CD226)	DNAX Accessory Molecule, PTA1, TLiSA1, Platelet and T cell activation	Ig superfamily. Binds LFA-1, PVR, and nectin-2, NK cell immune surveillance and tumor cell cytotoxicity, facilitates NK/dendritic cell interaction, involved in	[76] [77] [78] [79] [80] [81–83]

Marker	Aliases	Function in NK cells	References
	antigen, T lineage-specific activation antigen 1 antigen	immune synapse formation and NK cell activation; soluble CD226 can reduce tumor cell growth.	
2B4 (CD244)	Natural Killer Cell Receptor 2B4, NAIL (NK Cell Activation-Inducing Ligand), NKR2B4, SLAMF4, h2B4, Nmrk	SLAM family of receptors, ligand for CD48, stimulates IL-2-activated NK cells; NK activating signals transmitted through CD244/SAP/Fyn pathway; inhibitory signals through CD244/EAT-2.	[84, 85] [86] [8]
NKG2D (CD314)	KLRK-1 (Killer Cell Lectin-Like Receptor Subfamily K, Member), KLR	C-type lectin family, binds MICA/B and ULBPs on target cells, pairs with DAP10; signals through PI3K/Akt and GRB2/VAV1; cytotoxicity	[8] [87–91] [92]
NKp46 (CD335)	Natural Cytotoxicity Triggering Receptor 1 (NCR1), LY94, NK cell Activating Receptor	NK cell activating receptor; Ig-like transmembrane receptor; binds viral hemagglutinin; signals lysis of tumor cells; triggers apoptosis of neutrophils in conjunction with Fas/FasL; no co-activation signal required.	[93] [94] [95] [96]
NKp30 (CD337)	Natural Cytotoxicity Triggering Receptor-3, Lymphocyte Antigen 117, Activating Natural Killer Receptor P30, MALS.	NK cell activating and Ig-like transmembrane receptor, CD28 family member; expressed on resting PB NKs; activation of NKs through ERK1/2; production of IFNγ; No co- activation signal required; important for NK cell/DC contact.	[38, 95, 96]
Perforin	Perforin 1 PFP (Pore Forming Protein), FLH2, PFN1, Cytolysin,	Contained in cytolytic granules; required for cytotoxicity. Similar mechanism to complement component C9.	[97–99] [23]

*Abbreviations: ADCC - antibody-mediated cellular cytotoxicity; DAP10 – DNAX-activating protein of 10 kDa; DC – dendritic cell; DNAX – tau subunit of DNA polymerase III; ERK – extracellular signal related kinase; EAT-2 Ewing's sarcoma-activated transcript-2; FHL – familial hemophagocytic lymphohistiocytosis; GRB – growth factor receptor-bound protein; HCMV – human cytomegalovirus; HIV – human immuno-deficiency virus; HLA – human leukocyte antigen; JAK – Janus kinase; KIR - killer Immunoglobulin-like receptors; LFA-1 – lymphocyte function associated antigen-1; MHC – major histocompatibility complex; MICA/B – MHC class I chain-related A and B proteins; NKAT- NK associated transcript; NKIS – NK immunologic synapse; PB – peripheral blood; PVR – polio virus receptor; SAP – SLAM-associated protein; SLAM – signaling lymphocyte activation molecule; STAT – signal transducer and activator of transcription.

Table 2

Antibody clones and experimental set up.

Tube number	Antibody 1	Antibody 2	Antibody 3	Antibody 4
1	CD3 PerCP BD 340663 Clone SK7	CD56 Alex Fluor 647 Catalogue # BD 557711 Clone B159	CD158a FITC Catalogue # BD556062 Clone HP-3E4	NKB1 (CD158e1) PE Catalogue # BD555967 Clone DX9
2	CD3 PerCP	CD56 AF647	CD158b FITC Catalogue # BD559784 Clone CH-L	CD8 PE Catalogue # BD555635 Clone HIT8a
3	CD3 PerCP	CD56 AF647	CD16 FITC Catalogue # BD555406 Clone 3G8	CD16 PE Catalogue # BD347617 Clone B73.1
4	CD3 PerCP	CD56 AF647	CD11a FITC Catalogue # BD555383 Clone HI111	CD2 PE Catalogue # BD555327 Clone RPA2.10
5	CD3 PerCP	CD56 AF647	CD94 FITC Catalogue # BD555888 Clone HP-3D9	Nkp46 (CD335) PE Catalogue # Beckman Coulter im3711 Clone BAB281
6	CD3 PerCP	CD56 AF647	DNAM1 (CD226) FITC Catalogue # BD559788 Clone DX11	NKG2D (CD314) PE Catalogue # R&D#fab139p Clone 149810
7	CD3 PerCP	CD56 AF647	CD11b Biotin Catalogue # BD555387 Clone ICRF44	Nkp30 (CD337) PE Catalogue # R&D#fab1849p Clone 210845
8	CD3 PerCP	CD56 AF647	CD244-FITC Catalogue # BD550815 Clone 2-69	CD27 PE Catalogue # BD555441 Clone M-T271
9	CD3 PerCP	CD56 AF647	CD122 Biotin Catalogue # BD554524 Clone Mik-β3	CD117 PE Catalogue # BD555714 Clone YB5.B8
10	CD3 PerCP	CD56 AF647	CD18 FITC Catalogue # BD 555923 Clone 6.7	CD57 PE Catalogue # BD 560844 Clone NK-1
11	CD3 PerCP	CD56 AF647	Perforin-FITC Catalogue # BD 556577 Clone deltaG9	
Control	CD3 PerCP	CD56 AF647	Mouse IgG – FITC Catalogue # BD 556577	Rat IgG - PE Catalogue # BD 559317
Control	CD3 PerCP	CD56 AF647	Mouse IgG – PerCP Biolegend Catalogue #400250	Mouse IgG - AF647 Catalogue # BD 557783
Streptavidin-conjugated antibody	Alexa Fluor 488-streptavidin Molecular Probes			

Table 3

Percent positive cells and mean fluorescence intensity of all markers in total NK cells, CD56^{bright}, and CD56^{dim} subpopulations.

	Total NK % positive mean +/− S.D. (range)	Total NK MFI mean +/− S.D. (range)	CD56^{bright} % positive mean +/− S.D. (range)	CD56^{bright} MFI mean +/− S.D. (range)	CD56^{dim} % positive mean +/− S.D. (range)	CD56^{dim} MFI mean +/− S.D. (range)
CD56^{+}, CD3^{-} N=40	**6.47** +/− **3.97** (0.61 – 16.87)		**8.56** +/− **6.90** (0.63 – 38.32)		**90.20** +/− **6.84** (61.68 – 98.29)	
CD2 N=36	**72.0** +/− **18.16** (23.1 – 100)	**268.03** +/− **177.4** (30.4 – 840)	**94.09** +/− **9.24** (66.7 – 100)	**692.8** +/− **448.95** (203 – 2325)	**69.35** +/− **19.55** (18.5 – 100) N=37	**227.59**+/− **153.62** (18.1 – 646)
CD8 N=37	**39.33** +/− **16.75** (8.92 – 77.3)	**43.01**+/− **58.11** (4.46 – 348)	**34.21** +/− **17.29** (0 – 70.8) N=36	**24.29**+/− **24.41** (4.02 – 99.7)	**38.66**+/− **17.83** (8.85 – 78.2)	**42.66** +/− **51.65** (4.07 – 299)
CD11a N=36	**90.35** +/− **16.18** (24.6 – 100)	**118.78** +/− **66.44** (29.9 – 311) N = 37	**98.78** +/− **5.02** (75 – 100)	**114.16** +/− **70.18** (25.5 – 331)	**92.2** +/− **12.5** (50.9 – 100) N=37	**118.5** +/− **65.5** (30.1 – 310)
CD11b	**80.01** +/−**24.48** (7.1– 99.4) N=37	**70.5** +/− **36.7** (9.22 – 183) N=37	**94.76** +/− **15.75** (14.3 – 100) N=31	**79.73** +/− **43.06** (18.4– 230) N=37	**79.87**+/− **23.35** (7.14 – 99.4) N=32	**65.18** +/− **42.02** (4.16– 180) N=38
CD16 3G8	**48.28** +/− **23.26** (4.8– 88.7) N=37	**81.08** +/− **104.56** (4.85 – 602) N=37	**26.32** +/− **20.98** (0 –100) N=37	**34.53** +/− **84.44** (2–519) N=37	**54.38** +/− **25.27** (4.2– 93.7) N=38	**85.38** +/− **106.1** (4.04– 605) N=38
CD16 B73.1	**53.68** +/− **21.6** (1.3– 85.9) N=37	**70.02** +/− **56.04** (6.49– 219)	**19.64** +/− **17.82** (0–57.9) N=37	**17.04** +/− **22.68** (2.19– 96.8)	**58.12** +/− **23.54** (1.79 – 92) N=38	**75.6** +/− **63.05** (3.49 – 227)
CD18	**90.89** +/− **13.03** (46.6– 100) N=22	**85.7** +/− **62.39** (21.6 – 265) N=21	**92.21** +/− **20.44** (20 – 100) N=23	**81.51** +/− **78.15** (6.42 – 287) N=22	**90.57**+/− **12.99** (43.2 – 100) N=23	**83.58** +/− **61.1** (19.9 – 263) N=22
CD27	**14.47** +/− **15.4** (1.8– 81.8) N=30	**10.72** +/− **7.94** (3.33 – 41.4) N=35	**33.5**+/− **14.74** (8 – 58.7) N=27	**24.96** +/− **21.18** (4.73 – 89) N=32	**10.74** +/− **9.73** (0.66– 46.8) N=31	**9.45** +/− **8.12** (3.0 – 43) N=36
CD57	**44.9** +/− **19.53** (17.2– 94.1) N=22	**757.7** +/− **493.28** (67.4 – 1738) N=22	**19.69** +/− **28.34** (0 –100) N=23	**113.62** +/− **183** (1.84– 560) N=23	**46.09** +/− **19.6** (18.5– 94.7) N=23	**792.0** +/− **538.86** (68.7 – 1876) N=23
CD94	**41.85** +/− **17.56** (3.3 – 82.4) N=32	**20.59** +/− **15.37** (6.5 – 88) N=37	**82.21** +/− **19.38** (50–100) N=30	**57.12** +/− **29.05** (14.1 – 121) N=34	**38.73** +/− **18.64** (8.4 – 82.6) N=33	**15.92** +/− **10.47** (5.3 – 64.1) N=38
CD117	**6.70** +/− **18.70** (0 – 89.33) N=22	**14.38** +/− **32.35** (2.13 – 162) N=27	**72.4** +/− **41.98** (0 – 100) N=18	**18.04** +/− **28.89** (0 – 107) N=23	**5.94** +/− **18.42** (0 – 89.1) N=23	**13.22** +/− **32.37** (2.03 – 169) N=28
CD122	**76.42** +/− **27.72** (0 – 100) N=30	**48.49** +/− **25.78** (3.06 – 126) N=35	**76.94** +/− **35.34** (0 – 100) N=29	**67.09** +/− **39.2** (0 – 178) N=34	**75.85** +/− **27.70** (0 – 99.97) N=31	**48.16** +/− **25.78** (3.04 – 124) N=36
CD158a N=37	**9.97** +/− **9.73** (0 – 46.86)	**7.67** +/− **8.30** (2.9 – 44.5)	**4.37**+/−**10.83** (0 – 50)	**5.35** +/− **6.76** (0 – 41.8)	**11.02** +/− **10.31** (0 – 51.26)	**7.94** +/− **8.98** (2.93 – 50.4)
CD158b N=37	**24.62** +/− **12.52** (0.34 – 63.4)	**13.49** +/− **7.32** (3.39 – 34.1)	**9.69** +/− **12.68** (0 – 50)	**7.44** +/− **6.02** (0 – 24.3)	**26.47** +/− **13.84** (0 – 69)	**14.07** +/− **7.72** (3.39 – 36.6)
NKB1 (CD158e1) N=37	**15.70** +/− **10.89** (0 – 37.7)	**40.87** +/−**35.65** (1.84 – 163)	**7.24** +/− **17.46** (0 – 100)	**11.43** +/− **15.90** (0 – 51.7)	**17.40** +/− **11.17** (0 – 35.7)	**44.51** +/− **39.98** (1.85 – 178)

	Total NK % positive mean +/− S.D. (range)	Total NK MFI mean +/− S.D. (range)	CD56^{bright} % positive mean +/− S.D. (range)	CD56^{bright} MFI mean +/− S.D. (range)	CD56^{dim} % positive mean +/− S.D. (range)	CD56^{dim} MFI mean +/− S.D. (range)
DNAM1	**33.28** +/− **25.31** (0 – 96) N=37	**12.28** +/− **9.38** (4.79 – 52.3) N=37	**47.25** +/− **33.53** (0 – 100) N=36	**20.52** +/− **39.10** (5.42 – 244) N=36	**32.60** +/− **26.22** (0 – 94.7) N=38	**10.82** +/− **6.45** (4.71 – 40.3) N=38
2B4	**35.06** +/− **27.73** (0.6– 96.4) N=30	**11.20** +/− **6.28** (4.61 – 38) N=35	**22.96** +/− **24.44** (0 – 100) N=36	**11.4** +/− **11.97** (5.42 – 244) N=36	**35.63** +/− **27.29** (2.47 – 97.9) N=31	**13.39** +/− **14.42** (4.61 – 89.5) N=36
NKG2D	**49.75** +/− **23.80** (0 – 86.92) N=37	**19.23** +/− **11.74** (4.89 – 55.3) N=37	**73.32** +/− **23.0** (0 – 100) N=36	**34.28** +/− **30.95** (8.35 – 195) N=36	**47.56** +/− **23.56** (0 – 86.5) N=38	**17.48** +/− **10.96** (4.27 – 56.3) N=38
NKp46	**43.25** +/− **26.0** (0.8 – 95.5) N=32	**22.32** +/− **14.02** (5.6 – 56.8) N=37	**76.43** +/− **25.84** (0 – 100) N=31	**49.37** +/− **34.34** (0 – 141) N=34	**40.5** +/− **26.8** (0 – 97.8) N=33	**20.3** +/− **13.45** (4.72 – 55.8) N=38
NKp30	**12.28** +/− **16.54** (0 – 84.9) N=31	**7.73** +/− **6.34** (1.83 – 24.9) N=36	**8.36** +/− **18.49** (0 – 100) N=31	**5.61** +/− **7.24** (0 – 116) N=36	**12.93** +/− **16.44** (0 – 84.3) N=32	**9.48** +/− **10.52** (1.85 – 56.6) N=37
Perforin	**61.37** +/− **20.74** (2.0– 91.6) N=36	**39.02** +/− **19.8** (8.94 – 77.0) N=40	**16.15** +/− **21.41** (0 – 100) N=35	**15.26** +/− **24.56** (0 –132) N=35	**68.34** +/− **19.11** (4.14 – 95) N=35	**41.94** +/− **19.78** (13 – 74.4) N=35