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Impact of blood processing variations on Natural Killer cell frequency, activation, chemokine receptor expression and function

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Abstract

Understanding the role of natural killer (NK) cells in human disease pathogenesis is crucial and necessitates study of patient samples directly *ex vivo*. Manipulation of whole blood by density gradient centrifugation or delays in sample processing due to shipping, however, may lead to artifactual changes in immune response measures. Here, we assessed the impact of density gradient centrifugation and delayed processing of both whole blood and peripheral blood mononuclear cells (PBMC) at multiple timepoints (2–24 hrs) on flow cytometric measures of NK cell frequency, activation status, chemokine receptor expression, and effector functions. We found that density gradient centrifugation activated NK cells and modified chemokine receptor expression. Delays in processing beyond 8 hours activated NK cells in PBMC but not in whole blood. Likewise, processing delays decreased chemokine receptor (CCR4 and CCR7) expression in both PBMC and whole blood. Finally, delays in processing PBMC were associated with a decreased ability of NK cells to degranulate (as measured by CD107a expression) or secrete cytokines (IFN-γ and TNF-α). In summary, our findings suggest that density gradient centrifugation and delayed processing of PBMC can alter measures of clinically relevant NK cell characteristics including effector functions; and therefore should be taken into account in designing clinical research studies.

Keywords

Natural Killer cells; flow cytometry; activation; delayed processing; PBMC; whole blood; ficoll; chemokine receptor

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1. Introduction

Quantitative measures of natural killer (NK) cell frequency, activation, trafficking potential and effector functions are important to consider in clinical research because these measures are associated with reduced disease pathogenesis of viral and neoplastic diseases (Orange and Ballas, 2006). Nonetheless, the study of human NK cells from diseased patients directly *ex vivo* has two important challenges. Firstly, in multicenter trials and developing country settings the time from venipuncture to sample processing may be prolonged due to long transport times to a central research laboratory. Secondly, there is little consensus on whether peripheral blood mononuclear cells (PBMC) and whole blood are equivalent sample types for studies of NK cells. These are important considerations in designing clinical studies because it is often unfeasible to isolate PBMCs from whole blood in a timely manner. Thus, delays in processing and the type of starting sample may affect measurements of NK cell characteristics; however, their impact has not been well characterized.

Previous studies have shown that PBMC prepared by density gradient centrifugation may not be equivalent to whole blood for measuring some leukocyte parameters. Specifically, density gradient centrifugation altered the proportion of T-cells expressing cytokine receptors (varying by specific receptor), and increased the proportion of T-cells expressing adhesion molecules (Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003). Likewise, delays in the time from venipuncture to sample processing altered the phenotype or functional responses of leukocytes. Ekong and colleagues found that delays in processing PBMC reduced T-cell frequencies (Ekong et al., 1993) and others have reported impaired T-cell responses as measured by cytokine secretion or proliferation (Betensky et al., 2000; Bull et al., 2007; Kierstead et al., 2007; McKenna et al., 2009; Weinberg et al., 2009). As a potential mechanism to explain these findings, McKenna and colleagues (McKenna et al., 2009) recently demonstrated that delayed processing of blood increased the frequency of activated CD11bpos and CD15pos granulocytes, and that these leukocytes inhibited T-cell responses. Furthermore, the induction of changes in activation and functional responses is not confined to T-cells. Delayed processing also reduced monocyte and dendritic cell responses to Toll like receptor ligands (Meier et al., 2008). Thus, density gradient centrifugation and delayed processing of blood or PBMC can affect important leukocyte parameters; however, their effects on NK cells have not been well described.

Here we report the impact of density gradient centrifugation and delayed processing on NK cell frequency, activation, chemokine receptor expression (as markers of trafficking potential) and effector function in whole blood and PBMC at multiple timepoints (2–24 hrs.). Our results suggest that while delayed sample processing does not affect NK cell frequencies, both delayed processing and density gradient centrifugation alter chemokine receptor expression and *in vitro* effector functions. It is therefore crucial to take these factors into account in designing clinical studies that measure innate immune responses.

2. Materials and Methods

2.1 Study Subjects

Blood samples from a total of 11 adult (20–31 years) females enrolled in an observational cohort of healthy women at Edendale Hospital (a district level hospital in Pietermaritzburg, KwaZulu Natal, South Africa) were included in these studies. Participantsgave informed consent. The University of KwaZulu Natal Biomedical Research Ethics Committee (E118/06), the Edendale Hospital ethics committee and the Massachusetts General Hospital Internal Review Board approved this study.

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2.2 Assessment of NK cell frequency, activation and chemokine receptor expression in whole blood and PBMC

A total of 34 mls of blood was drawn into four acid citrate dextrose (ACD) tubes (BD Biosciences), transported by vehicle at atmospheric temperature (23–25°C) to the research laboratory in Durban, South Africa, and kept at ambient room temperature (20–23°C) until being processed. All donors were bled within 15 minutes of each other and all samples reached the laboratory within two-hours of venipuncture. At 2, 8,16 and 24 hours after venipuncture, PBMC were prepared by density gradient centrifugation using Histopaque 1077 (Sigma, St Louis, MO) as per the manufacturers protocol. Whole blood (processed at 2 and 24 hours after venipuncture) and PBMC (processed at 2, 8,16 and 24 hours after venipuncture) were stained using separate panels of antibodies to measure NK cell frequency, activation and chemokine receptor expression by multiparametric flow cytometry on an LSRII flow cytometer (BD Biosciences).

For whole blood staining, 300μl whole blood was stained with three separate antibody cocktails for 20 minutes at 4°C in the dark. Subsequently, red blood cells were lysed with 1ml Versalyse Lysing Solution (Beckman Coulter, France) and the cells were concomitantly fixed with IOTest3 (Beckman Coulter, France), per the manufacturers protocol for the concomitant ‘Fix and Lyse’ procedure.

For PBMC staining, two million PBMC per staining condition were washed and resuspended in 50μl of calcium and magnesium free Dulbecco’s phosphate buffered saline (DPBS) (Invitrogen). The cells were then stained for 20 minutes with LIVE/DEAD fixable aqua dead cell stain (Invitrogen) per the manufacturers instructions. Next, the samples were washed, resuspended in DPBS, and stained with each of three separate antibody cocktails for 20 minutes at 4°C in the dark. The cells were then washed twice to remove excess antibody and fixed with 1% paraformaldehyde (Sigma, St Louis, MO) before being acquired on the LSRII.

Antibody cocktails for staining panels—All antibodies were purchased from BD Biosciences unless otherwise indicated. The antibody cocktails consisted of anti-CD3 Qdot-605 (Invitrogen) or anti-CD4 Qdot-605 (Invitrogen), anti-CD8 Qdot-655 (Invitrogen), anti-CD14 Pacific Orange (Invitrogen), anti-CD19 Pacific Orange (Invitrogen), anti-CD16 Pacific Blue (Invitrogen), anti-CD38 PE-Cy7 or anti-CCR7 PE-Cy7, anti-CD56 PE-Cy5, anti-CD69 FITC or anti-CCR4 FITC or anti-NKp46, anti-HLA-DR APC-H7, anti-CD69 APC or anti-CCR5 APC, anti-CD45 AlexaFluor 700 (Invitrogen)-for whole blood staining only or anti-CD3 AlexaFluor 700 (Invitrogen) conjugated antibodies. All antibodies were titrated to determine optimal saturating concentrations for staining.

2.3 Stimulation of PBMC for the assessment of NK cell degranulation and intracellular cytokine expression

One million PBMC were cultured in 1 ml of RPMI 1640 (supplemented with 10% fetal calf serum and 1% Penicillin/streptomycin), 0.5 μl Golgistop (BD Biosciences), 5μg/ml brefaldin A (SIgma, St Louis, MO), and simultaneously stained with 10μl anti-CD107a PE-Cy5 (BD Pharmingen). After 4 hours, Phorbol-12-myristate-13- acetate (PMA) (Sigma, St Louis, MO) plus Ionomycin (Sigma, St Louis, MO) were added at 1μg/ml and 0.5 μg/ml, respectively, and incubation continued for a further 2 hours at 37°C, 5% CO_2. After a total of 6 hours of incubation cells were washed twice with DPBS and stained for 20 minutes with LIVE/DEAD fixable aqua dead cell stain (Invitrogen) per the manufacturers instructions. After being washed twice, cells were surface stained for 20 minutes at 4°C in the dark with anti-CD3 AlexaFluor 700 (Invitrogen), anti-CD14 Pacific Orange (Invitrogen), anti-CD16 Pacific Blue (Invitrogen) and anti-CD56 PE (BD Biosciences) conjugated

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antibodies. Next, the cells were washed with DPBS and fixed with 100μl Fix&Perm Reagent A (Invitrogen) for 20 minutes at room temperature. Following fixation, cells were washed and incubated in 100μl Fix&Perm Reagent B (Invitrogen) containing anti-Interferon-γ PE-Cy7 and anti-tumour necrosis factor-α FITC conjugated antibodies, for a further 20 minutes at 4°C in the dark. Finally, cells were washed, resuspended in DPBS and 500,000–1000,000 events were acquired on the LSRII. In addition, eight-peak mid-range beads (BD Biosciences) were used to standardize instrument settings on the LSRII between each run.

2.4 Analysis of flow cytometric data

The proportions of activated, chemokine receptor-expressing, degranulating, or cytokine secreting NK cells were analyzed using Flowjo (v9.0.1, Treestar). Phenotypically, NK cells were defined as aqua viability dye negative (live), CD3neg, CD14neg, CD19neg, CD16pos and/or CD56pos. In the gating strategy, gates were standardized using fluorescence minus one (FMO) controls. Time and singlet gates were included. We assessed both proportions of NK cells and levels of marker expression as measured by mean fluorescence intensity (MFI). All measures of frequency and activation were done in duplicate using independent flow cytometric panels. Means for duplicate measures have been indicated.

Data were summarized and statistical tests performed in Graphpad Prism (v5, Graphpad Prism Inc.). Paired t-tests were applied to assess statistical significance of differences between different timepoints, or between different sample-types from an individual. A repeated measures ANOVA was used to assess statistical significance of differences between several (>2) timepoints. To test for linear trend, a test for trend was calculated, and where relevant we report the p value testing for nonlinear variation after correcting for a linear trend. P-values were not corrected for comparisons that we defined *a priori.*

3. Results

Previous studies have shown that delays in blood sample processing or the process of isolating PBMC may alter phenotypes or functions of leukocytes(Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003)(Betensky et al., 2000; Bull et al., 2007; Kierstead et al., 2007; McKenna et al., 2009; Weinberg et al., 2009). To quantify the impact of density gradient centrifugation and delayed processing on NK cells, we evaluated whole blood and PBMC samples from five healthy adults processed at different timepoints after venipuncture(Fig. 1a). These timepoints corresponded to processing of samples immediately on receipt (2 hrs), at the end of a routine working day (8 hrs), after an overnight rest (16 hrs), or one full day later (24 hrs). Using multiparameter flow cytometry, NK cells were defined based on the absence of T-cell, monocyte, or B-cell markers (CD3, CD14, and CD19, respectively), and the presence of CD16 and/or CD56 (Fig. 1b). In addition, non-viable cells and cell doublets were excluded from analysis.

3.1 Frequencies of NK cells are not significantly affected by delays in blood sample processing

Following delays in the preparation of PBMC from whole blood at 2, 8, 16, and 24 hours post-venipuncture, the frequencies of NK cells remained unchanged; however, there was a weak trend towards reduced frequencies of NK cells with time (repeated measure ANOVA p=0.147, post test for trend p=0.031, r^2=0.1) (Fig 2a). Likewise, the frequencies of NK cells, as a proportion of all CD45pos cells in whole blood, were similar in blood processed at 2 hours and 24 hours post venipuncture (means of 10.3% and 9.1% respectively), (Fig 2b). Whole blood was not assessed at 8 or 16 hours post venipuncture due to limited sample availability. Overall, we found no change in NK cell frequencies over time in both whole blood and PBMC. Based on previous reports of lymphocyte alterations attributed to sample

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processing (Renzi and Ginns, 1987;Tamul et al., 1995;Lin et al., 2002;Berhanu et al., 2003), we next wanted to assess whether density gradient centrifugation affected the quality of the NK cells.

3.2 Density gradient centrifugation alters NK cell activation and the expression of chemokine receptors

Using multi-parametric flow cytometry, we measured the activation status of NK cells *ex vivo*and the expression of selected chemokine receptors in the samples processed from the same five donors used previously (Fig 3). First, to assess NK cell activation we quantified the expression of CD38, CD69, HLA-DR and NKp46 on NK cells in whole blood vs. PBMC prepared 2 hrs after venipuncture (Fig 3a). We chose these markers for two reasons. On NK cells, the levels of CD69 and HLA-DR expression have previously been used as surrogates for acute and chronic activation, respectively (Ziegler et al., 1993;Fogli et al., 2004;Ravet et al., 2007). Secondly, we and other investigators have reported a loss of NKp46 expression on activated NK cells (Wong et al.; Fogli et al., 2008); thus, the level of NKp46 expression may also serve as a surrogate measure of NK cell activation.

The proportions of NK cells expressing CD69, a marker of acute activation, roughly doubled from 0.36% in whole blood to 0.85%, following density gradient centrifugation (p=0.034) (Fig 3a). There was, however, no change in the proportions of NK cells expressing HLA-DR. This increase in CD69 expression was not associated with a significant decrease in the proportion of NKp46 expressing NK cells (Fig 3a). In addition, we observed a modest, but statistically significant decrease in the proportion of NK cells expressing CD38 following density gradient centrifugation (69.78% in whole blood vs. 65.5% in PBMC, p=0.026). These results suggest that NK cells may be acutely activated by density gradient centrifugation; however, the degree of this effect was small.

Unlike some activation markers, chemokine receptors undergo rapid internalization and endocytic recycling, and therefore, have high rates of turnover at the cell surface (Neel et al., 2005). We therefore predicted that the cell surface expression of chemokine receptors might be more susceptible to the effects of density gradient centrifugation than activation markers. To test this hypothesis we quantified the expression of two chemokine receptors on NK cells in whole blood vs. PBMC (Fig 3b). We chose to specifically assess CCR4 and CCR7since both are expressed on NK cells and have ligands that are critical for trafficking to the skin and lymph nodes, respectively (Berahovich et al., 2006). With density gradient centrifugation we found a substantial increase in the average proportion of CCR4-expressing NK cells (10.5% in whole blood vs. 50.9% in PBMC, p<0.001) (Fig 3b). However, we observed a tendency for proportionally fewer NK cells expressing CCR7 (37.5% in whole blood vs. 21.8% in PBMC) following density gradient centrifugation, but this difference was not statistically significant (p=0.13). Consistent with our findings of increased proportions of CCR4-expressing NK cells, we also observed a tendency towards increased surface expression of CCR4 following density gradient centrifugation (2.7 $\log_{10}$ MFI in whole blood vs. 3.24 $\log_{10}$ MFI in PBMC, p=0.077). Likewise, we observed a statistically significant reduction of CCR7 expression on NK cells following density gradient centrifugation (3.1 $\log_{10}$ MFI in whole blood vs. 2.61 $\log_{10}$ MFI in PBMC, p=0.014) (supplementary table 1). Taken together, these data suggest that density gradient centrifugation marginally alters the activation status of NK cells, but dramatically alters their expression of chemokine receptors such as CCR4.

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3.3 Processing of blood beyond 8 hrs after venipuncture results in activation of NK cells and alteration of chemokine receptor expression

Based on our findings of changes in activation and chemokine receptor expression profiles following density gradient centrifugation, we next wanted to know how delays in processing, affected these parameters. Hence, we measured these parameters on NK cells in PBMC at 2, 8, 16 and 24 hrs after venipuncture and in whole blood at 2 and 24 hrs after venipuncture (Fig 4).

Similar to our previous findings, we observed changes in the expression of activation markers and chemokine receptors. We found no significant change in the proportions of NK cells in PBMC expressing either CD38 or HLA-DR with delays in processing (Fig 4a). However, we observed greater proportions of NK cells expressing CD69 ($p=0.027$) and lower proportions of NK cells expressing NKp46 in PBMC processed more than 8 hrs after venipuncture ($p<0.001$) (Fig 4a). The magnitude of these effects was greater than the comparison between whole blood and PBMC. Consistent with these changes, we also noted an increase in the MFI of CD69 and decrease in the MFI of NKp46 (supplementary table 1). Thus, delays in processing activated NK cells in PBMC and substantially reduced the proportions of NK cells expressing either CCR4 ($p<0.0001$) or CCR7 ($p=0.0008$) (Fig. 4b). However, these decreases were only noted in PBMC processed more than 8 hrs after venipuncture. Consistent with these findings, over time we also noted lower MFIs for both chemokine receptors (supplementary table 1). Finally, to address whether these effects were limited to NK cells we further analyzed measures of activation and chemokine receptor expression on T-cells in the same samples. Similar to our findings from NK cells, we observed a trend of increased activation for both CD4 and CD8 T-cells as measured by CD38 (supplementary Fig. 1), We also observed proportionally fewer CCR4, CCR5 and CCR7-expressing T-cells over time (supplementary Fig. 1). Thus the changes we observed were consistent for both T-cells and NK cells.

Likewise, in whole blood stained at 2 and 24 hrs after venipuncture, we observed no change in the proportions of NK cells expressing CD38, HLA-DR or CD69 (Fig 4c). In contrast, we found a decrease in the proportion of NKp46-expressing NK cells (42.6% at 2 hrs vs. 28.06% at 24 hrs, $p=0.009$) (Fig 4c). These findings were consistent with decreased surface densities of these receptors as measured by MFI (supplementary table 1). Furthermore, we discovered a dramatic loss of CCR7 staining on NK cells in whole blood stained at 24 hrs after venipuncture (37.5% at 2 hrs vs. 0.25% at 24 hrs, $p=0.008$), which was substantiated by a concomitant decline in the levels of CCR7 expression (supplementary table 1). In contrast, we found no difference in the proportions of CCR4-expressing NK cells (10.5% at 2 hrs vs. 6.8% at 24 hrs, $p=0.11$) (fig. 4d). Similarly, MFIs for CCR4 were only marginally lower at 24 hrs (2.70 MFI (log10) vs. 2.61 at 2 hrs. and 24 hrs., respectively, $p=0.04$; supplementary table 1). Taken together, these data suggest that delayed processing of whole blood minimally impacted NK cell activation status, but profoundly diminished expression of some chemokine receptors. Notably, these effects were more apparent with PBMC than whole blood.

3.4 Delayed processing of blood decreases functional responses of NK cells following in vitro stimulation

The significant loss of NKp46 expression in PBMC over time suggested that delayed processing could alter functional responses. Previously, De Maria and colleagues demonstrated diminished functional responses by NK cells with lower NKp46 expression (De Maria et al., 2003). To assess whether the changes we observed were accompanied by alterations in NK cell effector functions, we measured the impact of delayed sample processing on degranulation and cytokine secretion following *in vitro* stimulation (Fig 5).

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For these assays we selected 6 additional healthy donors. We used PMA plus Ionomycin to stimulate NK cells in PBMC prepared 2, 16 and 24 hrs after venipuncture (stimulation of PBMC prepared at 8 hrs was not performed due to insufficient sample availability). We quantified functional responses using flow cytometry by gating on NK cells expressing CD107a, a surrogate for degranulation (Alter et al., 2004), and interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α).

Following *in vitro* stimulation we observed a significant reduction in both NK cell degranulation and cytokine secretion with delays in processing that was most marked with delays of greater than16 hours (Fig 5). The proportion of NK cells expressing CD107a correlated with the proportion expressing IFN-γ ($p=0.002$, $r^2=0.59$), but not with the proportion expressing TNF-α. However, we noted a positive correlation between proportions of NK cells expressing IFN-γ and TNF-α ($p=0.008$, $r^2=0.37$). These findings suggest that delays in processing of PBMCs significantly impair NK cell cytokine secretion and degranulation.

4. Discussion

In clinical studies there are few standardized methods for assessing NK cell parameters, including their frequency, activation, chemokine receptor expression, or effector functions. Some investigators have reported marked differences in antiviral responses mediated by NK cells using fresh whole blood vs. PBMC prepared by density gradient centrifugation (Tiemessen et al., 2009). This finding along with previous reports of leukocyte alterations with density gradient centrifugation(Renzi and Ginns, 1987; Tamul et al., 1995; Lin et al., 2002; Berhanu et al., 2003) suggest that these factors are important to consider in designing studies using blood samples. Our work further extends these findings by quantifying the impact of sample type and processing delays on NK cells. For the first time we demonstrate that delays in processing alter chemokine receptor expression on NK cells and decrease NK cell functional responses.

Our overall aim was to assess the impact of density gradient centrifugation and delayed processing on NK cell frequency, activation, chemokine receptor expression and effector function. We found that NK cell frequencies in whole blood and PBMC were unaffected by delays in processing up to 24hrs. This is not unexpected since NK cells have a half-life of about 7–10 days *in vivo* (Zhang et al., 2007) so they would not be predicted to die or proliferate dramatically over a 24-hour period *ex vivo*. We also found that density gradient centrifugation acutely activated NK cells; however the magnitude of this change was relatively small and most likely biologically insignificant. In comparison, much greater alterations in acute activation of NK cells occur following vaccination (Horowitz et al., 2010). Similarly, we found slight differences in CD38 expression due to sample processing although the function of CD38 on NK cells remains undetermined. By using consistent sample processing methods these differences may be taken into account.

In contrast, the effects of density gradient centrifugation on chemokine receptor expression are dramatic, particularly for CCR4. Our results suggest that density gradient enhances the detection of CCR4 on NK cells. One possible explanation for this finding is that the reagents used in density gradient centrifugation may stabilize the cell-surface expression of highly labile chemokine receptors, Similar reported effects of enhanced chemokine receptor expression on T-cells further support this hypothesis(Berhanu et al., 2003). Thus, our results suggest that chemokine receptor expression is not comparable between NK cells in whole blood and PBMC. Using different sample types for comparisons may yield inconsistent results.

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In addition, delays in processing for more than 8 hours dramatically affect chemokine receptor expression on NK cells. This finding is consistent with similar reported effects on T-cells (Nicholson et al., 1984; Ekong et al., 1993; Betensky et al., 2000; Lin et al., 2002; Berhanu et al., 2003; Bull et al., 2007; Meier et al., 2008; McKenna et al., 2009). With NK cells, the expression of both CCR4 and CCR7 diminishesover time. This effect on CCR4 expression is most notable in PBMC; whereas, the effect on CCR7 expression is most notable in whole blood. Taken together, our findings imply that clinical samples must be analyzed within 8 hours of collection to reliably assess chemokine receptor expression on NK cells. Such guidelines are useful considerations for the design and implementation of clinical studies that quantify NK cell subsets.

Similarly, we found that delays of more than 16 hours significantly impaired degranulation and cytokine secretion by NK cells. These findings are consistent with previously described changes in functional responses by T-cells, dendritic cells and monocytes(McKenna et al., 2009; Meier et al., 2008). Notably delays of more than 8 hours severely impair dendritic cell and monocyte cytokine secretion following *in vitro* stimulation (Meier, 2008 #13;). Thus, 8 hours is a general benchmark for quality control assessment in deciding the usability of blood samples for measuring NK cell functional responses. While the mechanisms underlying dysfunction remain unknown, we speculate that, as previously shown for T-cells, the expansion of activated CD11bpos and CD15pos granulocytes may dampen NK cell responses through changes in the cytokine milieu (McKenna et al., 2009). Alternatively, intracellular signaling components required for robust responses may become depleted over time *ex vivo*. To understand the basis of this impairment further investigations are warranted; however, it is apparent that the time from sample collection to assay implementation is a critical consideration in obtaining valid results. This study extends the work of previous studies by providing an estimate of time for the assessment of NK cell parameters *ex vivo*. Samples that are analyzed greater than 16 hours after collection may yield deficient NK cell responses. Since we did not assess timepoints between 2and16 hours we cannot provide a more accurate estimate of a suitable limit on sample transport to permit functional analyses but based on the findings reported here, and those of others(McKenna et al., 2009; Meier et al., 2008), we believe 8 hours is a reasonable benchmark.

A few limitations of our study warrant mention. Firstly, our conclusions are limited to blood samples from adults. Lin and colleagues have reported different effects of sample processing variation using blood samples from young children and infants (Lin et al., 2002).Secondly, for these assays we used blood drawn on ACD treated tubes. It is plausible that blood drawn into ethylenediamine tetra-acetic acid (EDTA) tubes may yield different results, particularly for measures of activation. EDTA chelates divalent cations that are crucial for activation processes and thus may reduce the effects of *ex vivo* activation. Finally, since our cohort was small, there is risk of a type II error in interpretation of the data; hence findings of no effect should be interpreted with caution, and attention to possible trends should be made.

6. Conclusion

Based on these findings, interpretation of studies using NK cells processed after delays of greater than 8 hours or after density gradient centrifugation warrants careful consideration. In particular, variations in sample processing time and/or methods have the greatest impact on NK cell chemokine receptor expression and *in vitro* functional responses. Thus, where possible, delays in processing should be avoided, and careful attention should be made in the selection of NK cell parameters that are least affected by these variables in the assessment of clinical samples.

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Supplementary Material

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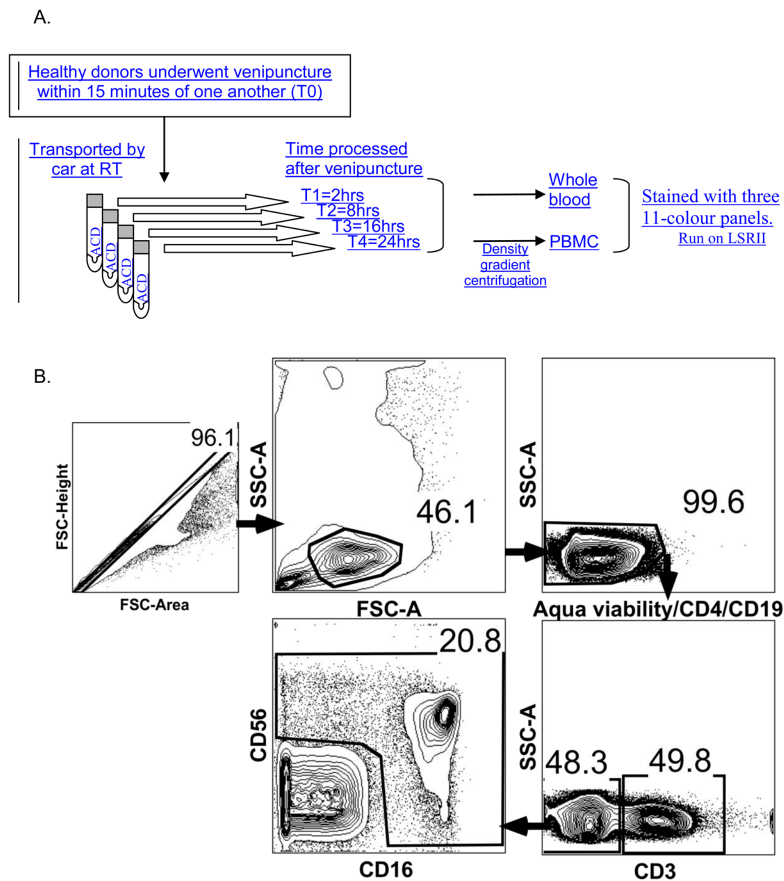

Fig 1. Study workflow

(A) Sample acquisition and processing time delays: A total of 34 mls of blood per individual was collected from five healthy adult female volunteers. The blood samples were collected into ACD tubes and subsequently processed at 2, 8,16 or 24 hrs after venipuncture. Peripheral blood mononuclear cells (PBMC) prepared by density gradient centrifugation and whole blood (WB) samples were surface stained with monoclonal antibodies, fixed and analyzed on an LSRII multiparameter flow-cytometer at the DDMRI, Durban, South Africa. (B) Gating strategy to define NK cell populations in PBMC (time, singlet, Live/CD14neg/CD19neg, CD3neg, CD56pos and/or CD16pos). For the analysis of whole blood, CD45 staining was used to identify lymphocytes (CD45pos).

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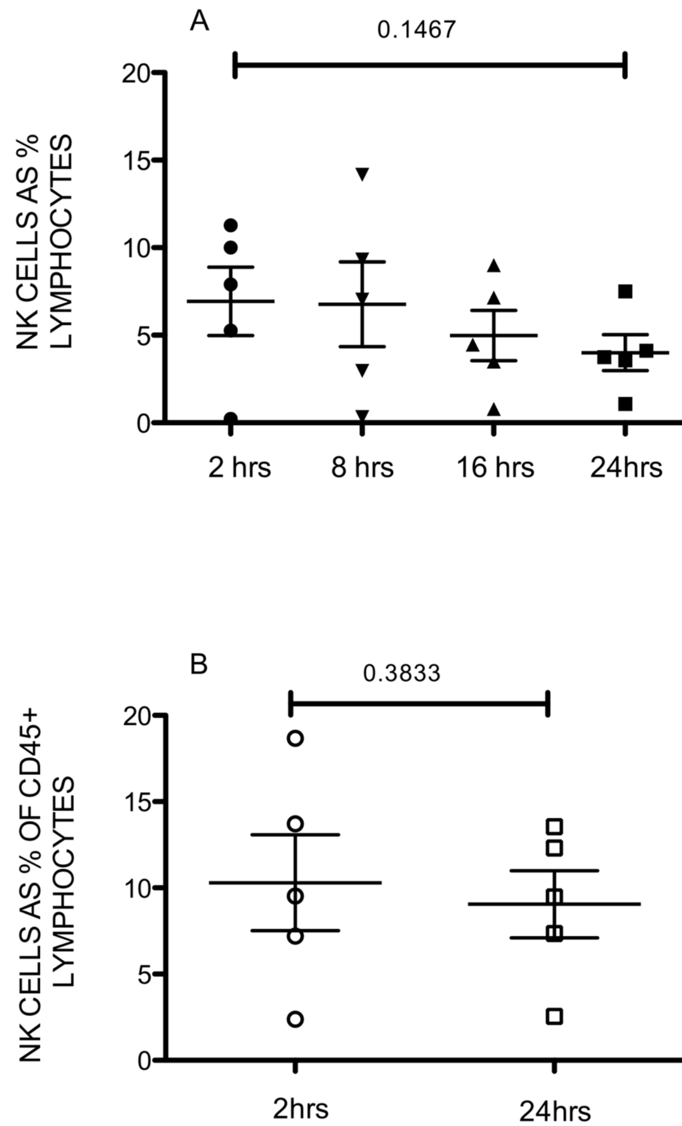

Fig 2. Frequencies of NK cells are not affected by delays in blood sample processing (n=5)

Fig 2A shows the frequency of NK cells as a proportion of lymphocytes in PBMC processed at 2, 8, 16 or 24 hrs (hrs) after venipuncture. The indicated p-value represents the result of a paired t-test. Fig 2B shows the frequency of NK cells as a proportion of CD45pos lymphocytes in whole blood processed at 2 or 24 hrs after venipuncture The indicated p-value represents the result of repeated measures analysis of variance (ANOVA). A post-test for linear trend gives p=0.031, r=0.312. Data shown represent means of duplicate measures for each participant (n=5). Open shapes (○ ▽ △□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 hours respectively, and as indicated. Each data point represents one individual.

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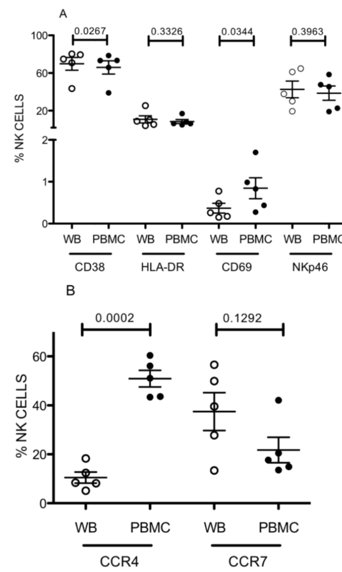

Fig 3. Density gradient centrifugation alters NK cell activationand the expression of chemokine receptors (n=5)

Fig. 3A shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in whole blood (WB) and PBMC processed 2 hrs after venipuncture. Fig. 1B shows the proportion of NK cells expressing CCR4 or CCR7 in WB and PBMC processed 2 hrs after venipuncture. The indicated p-values represent the results of paired t-tests. Data shown in Fig. 3A represent means of duplicate measures for each participant (n=5). Open shapes (○ ▽ △□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or dtri;), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 hours respectively, and as indicated. Each data point represents one individual.

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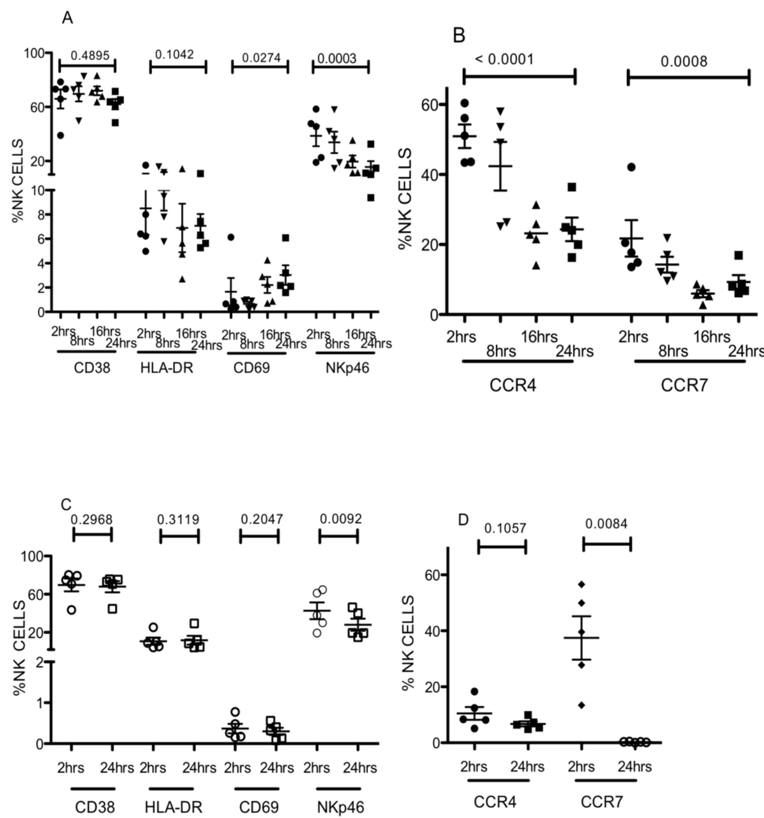

Fig 4. Delayed processing of blood alters NK cell activation and the expression of chemokine receptors (n=5)

Fig. 4A shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in PBMC processed after 2, 8, 16 or 24 hrs after venipuncture. Fig 4B shows the proportion of NK cells expressing CCR4 or CCR7 in PBMC processed after 2, 8, 16 or 24 hrs after venipuncture. The indicted p-values in Fig. 4A and 4B represent the results of repeated measures analysis of variance (ANOVA). Fig 4C shows the proportion of NK cells expressing CD38, HLA-DR, CD69 or NKp46 in whole blood (WB) at 2 or 24 hrs after venipuncture. Fig 4D shows the proportion of NK cells expressing CCR4 or CCR7 in WB at 2 or 24 hrs after venipuncture. The indicated p-values in Fig. 4C and 4D represent the results of a paired t-test. Data shown in Fig. 4A and 4C represent means of duplicate measures for each participant (n=5).Open shapes (○ ▽ △□) represent means of duplicate measures from whole blood, closed shapes represent means of duplicate measures from PBMC (■▼▲●); circles (● or ○), inverted triangles (▼ or ▽), upright triangles (▲ or △) and squares (■ or □) represent measures of NK cell frequency at 2, 8, 16 and 24 hours respectively, and as indicated. Each data point represents one individual.

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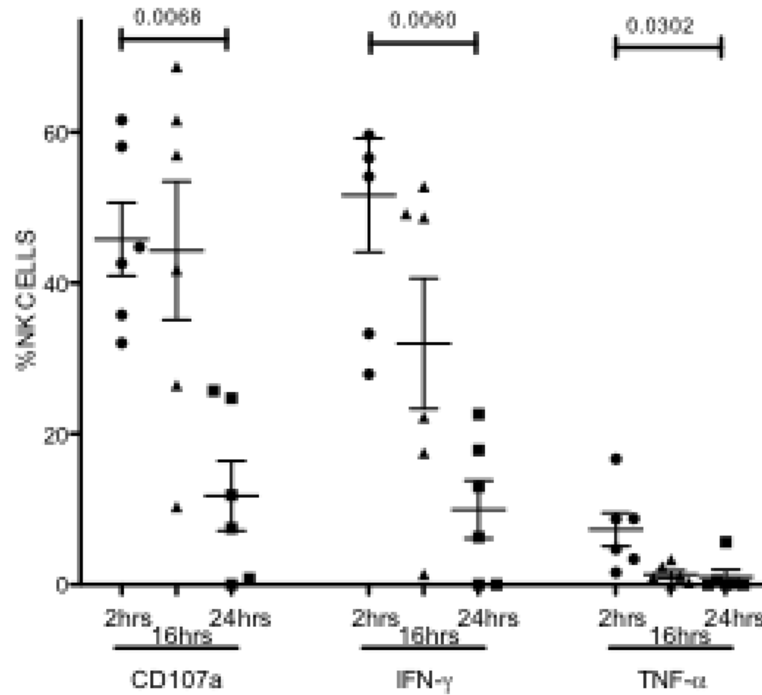

Fig 5. Delayed processing of blood decreases functional responses by NK cells as measured by CD107a expression and cytokine secretion following *in vitro* stimulation (n=6)

Fig. 5 shows the proportion of NK cells expressing CD107a (a surrogate for degranulation), interferon-γ (IFN-γ) or Tumour Necrosis Factor-α (TNF-α) in response to PMA/Ionomycin stimulation ofPBMC processed at 2, 16 or 24 hrs after venipuncture. The indicted p-value represents the results of repeated measures analysis of variance (ANOVA) (n=6). Circles, triangles and squares (■▲●) represent the NK cell parameter as denoted on the x-axis label, at 2, 16, and 24 hours respectively, and as indicated. Each data pointrepresents one individual.

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Table 1

NK cell readout	Parameter	Measure	Comparison Group 1	Comparison Group 2	Comparison Group 3	Comparison Group 4	Statistical comparison*
			WB	**PBMC**			
Activation	**CD38**	**% Of NK**	**69,78**	**65,95**			**0,03**
		MFI ($\log_{10}$)	3,34	2,99			0,13
	CD69	**% of NK**	**0,37**	**0,85**			**0,03**
		MFI ($\log_{10}$)	−0,54	1,64			0,07
	HLA-DR	% of NK	10,82	8,51			0,33
		MFI ($\log_{10}$)	**2,47**	**2,38**			**0,035**
	NKp46	% of NK	42,64	38,64			0,40
		MFI ($\log_{10}$)	**2,62**	**3,08**			**0,02**
Chemokine receptor	CC R4	**% of NK**	**10,47**	**50,92**			**0,0002**
		MFI ($\log_{10}$)	2,70	3,24			0,078
	CCR7	% of NK	37,46	21,76			0,13
		MFI ($\log_{10}$)	3,10	2,56			0,014
			WB 2hr	**WB 24hr**			
Frequency of NK		NK as % CD45 + lymphocytes	10,30	9,10			0,38
Activation	CD38	% of NK	69,78	67,92			0,30
		MFI ($\log_{10}$)	3,35	3,18			0,07
	CD69	% of NK	0,37	0,30			0,20
		MFI ($\log_{10}$)	2,49	2,50			0,50
	HLA-DR	% of NK	10,82	11,86			0,31
		MFI ($\log_{10}$)	−0,21	−1,80			0,21
	NKp46	**o/o Of NK**	**42,64**	**28,06**			**0,01**
		MFI ($\log_{10}$)	**2,62**	**2,39**			**0,03**
Chemokine receptor	CCR4	% of NK	10,47	6,77			0,11
		MFI ($\log_{10}$)	**2,70**	**2,61**			**0,04**
	CCR7	**% of NK**	**37,46**	**0,26**			**0,01**

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NK cell readout	Parameter	Measure	Comparison Group 1	Comparison Group 2	Comparison Group 3	Comparison Group 4	Statistical comparison*
		MFI (log_{10})	**3,10**	**1,60**			**<0,0001**
			PBMC 2hr	**PBMC 8hr**	**PBMC 16hr**	**PBMC 24hr**	
Frequency of NK		NK as % Lymphocytes	6,94	6,76	4,99	4,00	0,15
Activation	CD38	% of NK	65,95	69,66	71,89	62,03	0,49
		MFI (log_{10})	**2,96**	**3,45**	**3,16**	**2,92**	**0,04**
	CD69	**% of NK**	**1,66**	**0,63**	**2,21**	**3,03**	**0,03**
		MFI (log_{10})	**2,38**	**2,49**	**2,58**	**2,57**	**0,0019**
	HLA-DR	% of NK	8,51	10,00	6,90	7,06	0,10
		MFI (log_{10})	**1,67**	**2,02**	**−1,27**	**−1,65**	**<0,0001**
	NKp46	**% of NK**	**38,64**	**33,82**	**19,60**	**15,54**	**0,00**
		MFI (log_{10})	**3,08**	**2,99**	**2,71**	**2,60**	**<0,0001**
Chemokine receptor	CC R4	**% of NK**	**50,92**	**42,36**	**23,18**	**24,34**	**<0,0001**
		MFI (log_{10})	**3,24**	**3,00**	**2,67**	**2,69**	**0,01**
	CCR7	**% of NK**	**13,60**	**9,54**	**2,76**	**6,14**	**0,0008**
		MFI (log_{10})	**2,56**	**2,41**	**2,14**	**2,20**	**<0,0001**

* two-tailed t-tests or repeated measures ANOVA