Published in final edited form as:

Cytometry B Clin Cytom. 2017 January ; 92(1): 33–41. doi:10.1002/cyto.b.21485.

The Newborn Human NK Cell Repertoire is Phenotypically Formed but Functionally Reduced

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Abstract

Background—Infection is a leading cause of death worldwide in babies under 1 month of age. Better vaccines and therapeutics are desperately needed for this vulnerable population.

Methods—Because newborns rely heavily on the innate immune system, we evaluated cell phenotype and function of some of the earliest cellular responders during infection, natural killer (NK) cells. We used mass cytometry to provide a comprehensive comparison of NK cells from umbilical cord blood and adult peripheral blood.

Results—In unsupervised analyses, including viSNE and principal component analysis, the structure of the cord blood and adult NK cell repertoires are highly similar, distinguishable mainly by maturity-related markers expressed on rare subpopulations of cells. However, in functional analyses, cord blood NK cells show reduced degranulation and cytokine production following target recognition, as well as antibody-dependent cell-mediated cytotoxicity and apoptosis induction in targets.

Conclusions—These findings show that the structure of the NK cell repertoire is intact at birth, suggesting great potential for vaccine and therapeutic strategies targeting this cell population.

Key terms

natural killer cells; umbilical cord blood; mass cytometry; viSNE

About 2.7 million babies die each year in their first month of life, accounting for 45% of child deaths before the age of five. Infection is a leading cause of these deaths (1). Newborns do not have fully developed innate (2) or adaptive (3) immune responses, and current vaccine strategies are insufficient to fully mobilize these systems (4). The development of more effective vaccination techniques to protect this population will require a more thorough understanding of the structure of its immune defenses.

Because of limited antigen exposure in utero, newborns rely heavily on the innate system for protection (2). Natural killer (NK) cells, a component of this system, are among the earliest cellular responders to viral infection. Because their signaling paradigm is combinatorial, acting through a spectrum of activating and inhibitory receptors, NK cell phenotype and function are uniquely and closely linked. It is therefore critical to understand both aspects of this cell population in the newborn.

In adults, a spectrum of NK maturity is readily observable, and is canonically anchored by immature CD56^{bright} NKG2A⁺ and mature CD56^{dim}CD57⁺ cells (5,6). Additional receptors, especially the killer immunoglobulin-like receptors (KIR), provide further variation among mature NK cell subsets (7). Umbilical cord blood, a convenient source of cells representative of newborn circulation, has facilitated the comparison of newborn and adult NK cells. Cord blood NK cells have a less mature phenotype than adult NK cells, but the proportional abundance of the CD56^{bright} and CD56^{dim} subsets does not differ between these groups (8–10). Compared to adults, cord blood NK cells have been reported to express decreased or similar levels of adhesion molecules (8,10), and either decreased (9) or similar (8) levels of KIR expression.

Human cytomegalovirus (CMV) infection is associated with significant perturbations to the maturing NK cell repertoire, including expansion of a population of memory-like CD57⁺ NKG2C⁺ NK cells (11,12). CMV seropositivity is also associated with, although not required for, the development of a subpopulation of NK cells lacking the signaling adaptor FcR γ and/or the tyrosine kinase Syk (13–16). These cells appear to be specialized for antibody-dependent functions, including antibody-dependent cellular cytotoxicity (ADCC). Their abundance in cord blood has never been defined.

Functionally, cord blood NK cells are reduced in cytotoxicity when compared with adult NK cells, and this deficiency can be rescued via administration of exogenous cytokines, including IL-2, IL-12, IL-15, and IL-18 (8,17–21). Cytotoxicity has typically been measured by the release of ⁵¹Cr from target cells, providing a sensitive test of membrane permeability, but not an indication of apoptotic pathway initiation. Virus-specific responses have been tested primarily against HIV, where cord blood NK cells show reduced suppression (22) and ADCC (19).

A more thorough understanding of cord blood NK cell phenotype and function is of particular interest as cord blood is increasingly used in clinical settings. Cord blood has great potential utility in hematopoietic stem cell transplantation. Compared to adult bone marrow, cord blood contains more hematopoietic progenitor cells, requires less stringent human leukocyte antigen (HLA) matching, and is associated with lower incidence of graft-versus-host disease (23). It can also be rapidly obtained without risk to the donor. These properties are also increasingly being harnessed in immunotherapeutic strategies (24).

Previous work comparing cord blood and adult NK cells has typically reported frequencies of one or several receptors at once. However, our recent work using high-dimensional mass cytometry identified the significant diversity of the NK cell repertoire based on 25+ receptors (7,25). This diversity is decreased in cord blood (26). These findings suggest that a

broader view may be more instructive. Therefore, to better understand their differences and similarities, we undertook the most thorough comparison of cord blood and adult NK cell repertoires to date. We sought to capture the structure of the repertoire by measuring a spectrum of activating and inhibitory receptors simultaneously using high-dimensional mass cytometry. In addition, we confirm and extend previous comparative functional studies using updated techniques. This work therefore provides a unified, systems-level view of both biologic and therapeutic relevance.

MATERIALS AND METHODS

Subjects

Adult and cord blood samples used in mass cytometry analyses have been described (26). Briefly, the adult donors consisted of twelve healthy volunteers, 6M/6F, aged 22–45 years. Though six timepoints were collected for the original study, a single representative timepoint was used for these analyses. Additional adult samples for functional studies were collected from leukoreduction system chambers purchased from the Stanford Blood Bank. Cord blood samples were collected from healthy singleton pregnancies at Stanford Hospital. All samples were separated via Ficoll density gradient centrifugation, and either peripheral or cord blood mononuclear cells were cryopreserved. Samples used in the same experiment were thawed and stained simultaneously. All samples were collected under protocols approved by the Institutional Review Board of Stanford University.

Mass Cytometry Analyses

Detailed staining protocols have been described (26–28). Briefly, cells were stained with 25 mM cisplatin (29) for 1 min and quenched with 100% serum. They were stained with surface antibodies for 30 min on ice, fixed (BD FACS Lyse), permeabilized (BD FACS Perm II), and stained with intracellular antibodies for 45 min on ice. Cells were suspended in iridium interchelator (DVS Sciences) + 2% paraformaldehyde in phosphate-buffered saline (PBS) overnight. They were washed 1× in PBS and 2× in H₂O before acquisition on a mass cytometer (Fluidigm). The staining panel is shown in Supporting Information Table S1. Antibodies were conjugated using MaxPar X8 labeling kits (DVS Sciences). FCS files were normalized using Normalizer v0.1 MCR (MATLAB Compiler Runtime) (30).

viSNE

viSNE was implemented in MATLAB version R2013b as described (31). The 2800 NK cells were sampled from each donor for each of 12 adults and 12 cord bloods. The 26 markers shown in Figure 3B were used for dimensionality reduction. The algorithm was run in parallel for all donors. Data were exported to R for visualization.

Principal Component Analysis

PCA was conducted on a random sample of 1000 NK cells for each of adult and cord blood populations using the ade4 package in R. 10× repeated analysis on varying subsamples did not change the results.

Additional Supporting Information may be found in the online version of this article.

NK Cell Degranulation and Cytokine Production

To assess the ability of NK cells to directly recognize and respond to target cells by degranulating and producing cytokines, PBMCs were mixed with 721.221 target cells at an effector-to-target ratio of 1:1 in the presence of brefeldin A (eBioscience), monensin (eBioscience), and anti-CD107a-APC-H7 (H4A3, BD Biosciences) in 96-well plates. PMA/ionomycin cell stimulation cocktail (eBioscience) was used as a positive control for CD107a and cytokine production. Cells were incubated for 3 h at 37°C with 5% CO₂.

Antibody-dependent Cellular Cytotoxicity

PBMCs were mixed with Raji target cells at an effector-to-target ratio of 10:1 in the presence of 0.1–0.125 µg mL⁻¹ anti-CD20 mAb (non-fucosylated Rituximab, Invivogen), brefeldin A, monensin, and anti-CD107a-APCH7 in 96-well plates. PMA/ionomycin cell stimulation cocktail was used as a positive control for CD107a and cytokine production. Cells were spun at 300 g for 3 minutes, then incubated for 4 h at 37°C with 5% CO₂.

Cytotoxicity Assay

NK cells were purified from PBMCs via negative selection with NK Cell Isolation Kit (Miltenyi Biotec) and LS Columns (Miltenyi Biotec), and then mixed with 721.221 target cells labeled with TFL4 (OncoImmunin, Inc.) at an effector-to-target ratio of 1:1, 5:1, 10:1, and 20:1 in 96-well plates. Cells were spun at 100 g for 3 min, resuspended in PanCyToxiLux substrate (On-coImmunin), and incubated for 4 h at 37°C with 5% CO₂. Cytotoxicity was measured as the background-subtracted percentage of FITC⁺ 721.221 targets.

Fluorescent Staining and Flow Cytometric Analysis

Following incubation, EDTA (Hoefer) was added at 20 mM to arrest stimulation. Cells were surface stained with LIVE/DEAD Fixable Yellow Stain (Life Technologies), fixed and permeabilized with FACS Lysing Solution and FACS Permeabilizing Solution II (BD Biosciences), and stained with the following intracellular antibodies: CD3-PE (UCHT1, Biolegend), TNF-FITC (MAb11, BD Biosciences), IFN γ -V450 (B27, BD Biosciences), CD16-PerCP Cy5.5 (3G8, Biolegend). For degranulation, cytokine production, and ADCC experiments, cells were also surface stained with CD56-PE-Cy7 (B159, BD Biosciences). For cytotoxicity experiments, no additional antibodies were added. Stained cells were analyzed on the MACSQuant Analyzer (Miltenyi Biotec). Analysis and compensation were performed using FlowJo (FlowJo, LLC).

Statistics

In Figures 2–5, data were found to be non-normal using the Shapiro–Wilk test. Therefore, nonparametric Mann-Whitney U tests were used to compare cord blood and adult samples. In Figure 2, the Bonferroni correction was used to correct for multiple comparisons.

RESULTS

Unsupervised Approaches for Comparing Cord Blood and Adult NK Cells

We used mass cytometry to measure 41 parameters on peripheral blood mononuclear cells (PBMC) from 20 cord blood samples and 12 healthy adults. A serial gating strategy (Supporting Information Figs. S1 and S2) identified NK cells (26) for use in subsequent analyses.

Effective analysis of this data requires an analytical approach capable of harnessing its dimensionality. We therefore used unsupervised learning as an unbiased method of capturing the complexity of the NK repertoire. viSNE, a visualization tool for single-cell data, was used to project the multi-dimensional relationships among markers in cord blood and adult NK cells onto two dimensions (31). To facilitate direct comparison between cord blood and adult, all donors were grouped in the same analysis. The added density plots allow visualization of the relative frequencies of cells in each plot region (Fig. 1).

This analysis revealed striking similarity in the structure of cord blood and adult NK repertoires: that is, very few plot regions are present in one group and absent in the other. The markers that best define NK cells, CD56 and CD16, are highly expressed in both groups. However, cell density for adults is higher in regions associated with the expression of markers of maturity, such as CD57. Cord bloods, in contrast, have higher cell density in regions associated with markers of immaturity, such as NKG2A (Fig. 1, Region 1). The cord bloods also express very low levels of NKG2C and therefore lack the most prominent mature NKG2C⁺ populations (Fig. 1, Region 2).

KIR expression is concentrated in small subpopulations (Fig. 1, Regions 3, 4, and 5). Consistent with expression of KIRs on mature NK cells, these regions are more densely populated in the adult than in cord blood, particularly for KIR3DL1 expression (Fig. 1, Regions 4–5). In contrast to the expression patterns of KIRs, expression of several activating receptors (NKp30, NKp46, 2B4, and DNAM-1) is not confined to specific regions of the repertoire. DNAM-1 expression is decreased in cord blood NK cells, confirming previous results (10), and 2B4 is more highly expressed. The inhibitory receptor PD-1 is expressed primarily on mature NK cells, while the inhibitory receptor LILRB1 is not regionally limited. Both of these markers are more highly expressed on adult NK cells. Cord blood NK cells express the signaling adaptor FcR γ and the tyrosine kinase Syk at high levels. In the adult population, the loss of Syk and FcR γ in some donors does not appear to be limited to a specific NK cell subpopulation, including CD57⁺ or NKG2C⁺ cells. These data indicate that the cord blood NK cells have a less mature but structurally similar phenotype compared with adult NK cells.

As a quantitative follow-up to the findings from this visualization, we manually gated individual NK cell populations and compared individual receptor frequencies within cord blood and adult NK cell populations (Fig. 2). Example gateings are shown in Figure 2a, with statistical comparisons in Figure 2b. Our findings confirm both previous results (8–10) and the results from Figure 1. Compared to adult NK cells, cord blood NK cells have significantly lower levels of the maturity marker CD57 ($P = 1.8 \times 10^{-8}$) and higher levels of

the immaturity marker NKG2A ($P = 9.2 \times 10^{-5}$) (Fig. 2b). Cord blood NK cells also have higher levels of 2B4 ($P = 0.002$) and lower levels of DNAM-1 ($P = 8.9 \times 10^{-9}$) and PD-1 ($P = 0.00013$) compared to adult NK cells. None of the other comparisons revealed significant differences between cord blood and adult samples after correcting for multiple comparisons. These results support the conclusions that while levels of some maturity-driven markers differ between cord blood and adult NK cells, their overall repertoire structure is not significantly different.

Having defined these repertoire similarities and differences, we next used Principal Component Analysis (PCA) to find the directions of maximum variance in the data. Following the trend observed in Figure 1, in this analysis cord blood and adult NK cells are not readily distinguishable (Fig. 3a). The top three principal components, which together explain 68.0% of the variance, are all driven primarily by a limited set of markers, especially CD16, CD56, and CD94 (Fig. 3b). These canonical NK cell markers are highly expressed in both groups (Figs. 1 and 2). Together, these analyses indicate that the differences between cord blood and adult NK repertoires are primarily limited to rare maturity-driven markers that do not affect the repertoire structure, confirming the results in Figure 1.

Cord Blood NK Cells Are Functionally Reduced

Next, we tested the ability of cord blood and adult NK cells to produce cytokines and degranulate in response to recognition of MHC I-deficient 721.221 cells. Compared to adult NK cells, cord blood NK cells degranulate significantly less (CD107a, $P = 0.016$, Fig. 4a), and produce significantly less IFN γ ($P = 0.032$, Fig. 4b) and TNF ($P = 0.016$, Fig. 4c).

We also tested cord blood NK cells' ability to perform ADCC. While degranulation did not reach significance in this assay (Fig. 4d), cord blood NK cells produce significantly less IFN γ ($P = 0.026$, Fig. 4e) and TNF ($P = 0.0043$, Fig. 4f) than adult NK cells.

Cord Blood NK Cells Show Reduced Efficacy in Killing Target Cells

The 721.221 and ADCC assays describe NK cell function, but do not directly measure cytotoxicity. We therefore assessed granzyme B and caspase-8 activity in 721.221 targets using the PanToxiLux system (Fig. 5). Cord blood NK cells killed fewer targets at E:T ratios ranging from 1:1 to 20:1, reaching significance at 5:1 ($P = 0.010$, Fig. 5b) and 10:1 ($P = 0.0037$, Fig. 5c). Cord blood NK cells are thus less effective than adult NK cells at initiating cell death in their targets.

DISCUSSION

Here we use mass cytometry to provide a comprehensive comparative analysis of phenotype and function in cord blood and adult NK cells. We show that these groups share many phenotypic characteristics. The adult NK repertoire is denser in regions representing mature cells, while cord blood repertoires are denser in immature regions, but nearly all subpopulations are represented in both groups. The two groups cannot be separated by PCA, indicating that rare maturity-driven markers appear to constitute their differences. Following target recognition, cord blood NK cells degranulate less and produce less IFN γ and TNF compared to adults. They are also deficient in ADCC, as well as in inducing apoptosis in

target cells. Together, the findings presented here provide a resource to better understand NK and PBMC development and maturation. These data will have applications in understanding hematopoietic stem cell transplants, as well as in vaccine and therapeutic development.

Previous work revealed that cord blood NK cells are reduced in cytotoxicity (8,17–21). Our work extends these findings by measuring Granzyme B's delivery of a lethal hit to targets and cleavage of caspase-8. This indicates that cord blood NK cells are deficient not only in inducing a loss of membrane permeability in target cells, but also in the activation of this specific biochemical pathway. This defect can be rescued by cytokine administration (8,17–21), but the specific pathways compromised remain the subject of ongoing investigation. Previous studies have suggested that signaling through the activating receptor 2B4 may actually be inhibitory at the earliest stages of NK cell differentiation, due to a lack of expression of its adapter protein, known as signaling lymphocytic activation molecule associated protein (SAP) (32). However, we detected similar intracellular protein levels of SAP in cord blood and adult NK cells (data not shown).

Recently, “memory-like” $\text{FcR}\gamma^-$ NK cells that have enhanced antibody-dependent functional activity have been described (13–16). The suggested model of $\text{FcR}\gamma^-$ NK cell development, derived from data on adult samples, is that NK cells at varying stages of maturation can develop into $\text{FcR}\gamma^-$ cells (16). Our discovery that Syk and $\text{FcR}\gamma$ are expressed nearly universally in cord blood NK cells aligns with this idea (33). We further show that on a population level, $\text{FcR}\gamma^-$ NK cells do not appear to be defined by a specific surface phenotype, and are not limited to a particular subpopulation of NK cells. This raises the intriguing possibility that memory-like NK cells might arise in response to a variety of pathogenic and/or environmental triggers, not just human CMV infection.

Mass cytometry has allowed a much broader investigation of immune phenotype and function than was previously possible. The 41-parameter panel used in this study provides a comprehensive comparison of cord blood and adult NK cells, allowing for conclusions not only on differences in individual receptor expression, but also on the structure of the repertoire. Future mass cytometry studies should focus on functional analyses of cord blood NK cells, especially the signaling pathways involved in the cytotoxicity defect, to better understand potential drug targets.

In eliciting an immune response in newborns, mobilizing all arms of the immune system may provide a more effective approach than relying solely on immature adaptive responses. Our findings show that as a whole, cord blood and adult NK repertoires are similarly structured. The markers that distinguish the cord blood and adult repertoire are rare. This data, along with previous studies indicating that functional defects can be rescued by cytokines, may provide clinical promise. That NK cells in the newborn are not fundamentally different from adults, and have the potential for optimization, provides promise that they may be able to be harnessed in vaccine and therapeutic strategies.

Acknowledgments

The authors thank N. Bayless and M. Leipold for technical assistance.

Grant sponsor: National Science Foundation Training Grant; Grant number: DGE-114740; Grant sponsor: Ruth L. Kirschstein National Research Service Award; Grant number: 1F31AI118469-01; Grant sponsor: Beckman Young Investigator Award, NIH Directors New Innovator Award; Grant number: DP2AI11219301; Grant sponsor: 2013 Clinical Scientist Development Award; Grant number: 2013099; Grant sponsors: Doris Duke Charitable Foundation; McCormick Faculty Award.

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Fig. 1.

Structural similarity of cord blood and adult NK cell repertoires revealed by viSNE. viSNE plots for activating receptors, inhibitory receptors, maturation markers, and intracellular proteins for cord blood (**A**) and adult (**B**) NK cell repertoires. Expression of each marker is designated by color, from blue (low) to gold (high). Density plots (gray lines) visualize relative cell quantities in each region. $n = 12$ cord bloods, 12 adults, 2800 cells per donor.

Fig. 2. Manual gating and statistical comparisons confirm and extend viSNE findings. **(A)** Example gating of subpopulations of CD57⁺, NKG2A⁺, NKG2C⁺, KIR2DL1⁺, KIR2DL3⁺, NKp46⁺, Syk⁺, and FcRg⁺ NK cells in cord blood (top) and adults (bottom). **(B)** Statistical comparison of frequencies of individual receptors in cord blood (dark gray) and adult (light gray) NK cells. *P* values < 0.05 (Mann-Whitney U) are shown. *n* = 20 cord bloods, 12 adults. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 3.

Principal component analysis (PCA) identifies no separation between adult and cord blood NK cells. **(A)** First three PCA components, or axes which explain the most variance in the data, showing 1000 randomly selected cells from each of cord blood (blue) and adult (red) NK cells. Axes identify percent variance explained for each of the three components. **(B)** Loadings of first three PCA components. Horizontal bars represent relative contribution of each marker. Width of vertical bars is proportional to percent variance explained. $n = 20$ cord bloods, 12 adults, 1000 cells sampled from each group.

Fig. 4.

Cord blood NK cells are reduced in cytotoxicity and ADCC. CD107a staining (A, D), IFN γ production (B, E) and TNF production (C, F) following recognition of 721.221 target cells (A–C) or in Raji-Rituximab ADCC assay (D–F) for both cord blood (dark gray) and adult (light gray) NK cells. CD107a and cytokine frequencies are represented as background subtracted values. $n = 4$ adults and 5 cord bloods for 721.221 recognition; 6 cord bloods and 6 adults for ADCC. P values < 0.05 (Mann-Whitney U) are shown.

Fig. 5.

Cord blood NK cells are reduced in apoptosis induction in target cells. Pantoxilux cytotoxicity assay. FITC⁺ 721.221 targets indicate presence of Granzyme B and/or cleaved caspase-8. Effector:target ratios of 1:1 (A), 5:1 (B), 10:1 (C), and 20:1 (D) are shown. $n = 8$ cord bloods and 8 adults for 1:1 and 5:1; 7 cord bloods and eight adults for 10:1; 4 cord bloods and eight adults for 20:1. P values < 0.05 (Mann-Whitney U) are shown.