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Characterization of Natural Killer cells Expressing Markers Associated with Maturity and Cytotoxicity in Children and Young Adults with Sickle Cell Disease

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Abstract

Background: Sickle cell disease (SCD) is increasingly recognized as a red blood cell disorder modulated by abnormally increased inflammation. We have previously shown that in patients with SCD not on a disease modifying therapy (hydroxyurea or chronic transfusions) natural killer (NK) cell numbers are increased. In the current study we further investigated the NK cell function to determine if there was evidence of increased activation and cytotoxicity.

Procedure: We conducted a cross-sectional study of 44 patients with HbSS/HbS β^0 thalassemia at steady state (hydroxyurea=13, chronic transfusion=11, no disease-modifying-therapy=20) and 23 healthy controls. Using a fresh blood sample, NK immunophenotyping was performed as follows: NK cells (CD3-CD56+ lymphocytes) were evaluated for markers associated with activation (NKG2D, NKp30, NKp44, CD69) and maturity (CD57, Killer Immunoglobulin-like Receptors (KIR), CD56dim). Degranulation and cytotoxicity assays were performed to evaluate NK cell function.

Results: Patients with SCD who were not on disease modifying therapy had higher number of NK cells with an immunophenotype associated with increased cytotoxicity (NKG2D+, NKp30+, CD56dim+, and KIR+ NK cells) compared to healthy controls and patients on hydroxyurea. NK cells from SCD patients not on disease modifying therapy demonstrated significantly increased cytotoxicity (measured by assaying NK cell killing of the K562 cell line) compared to healthy controls ($p=0.005$). Notably, NK cell cytotoxicity against K562 cells in the hydroxyurea or chronic transfusion patients were not significantly different from healthy controls.

Conclusion: SCD is associated with increased NK cell function as well as increased NK cell numbers, which appears to be normalized with disease-modifying-therapy.

Keywords

Sickle cell disease; Hydroxyurea; NK cells

Introduction

Sickle cell disease (SCD) is an inherited red blood cell disorder caused by a mutation in the beta globin gene. This leads to hemoglobin polymerization in the deoxygenated state and chronic red cell hemolysis. SCD is also characterized as a hyper-inflammatory condition with increased neutrophil number, adhesion and activation¹⁻³. Monocytes are also activated and contribute to inflammation in SCD^{4,5}. More recently, other aspects of the innate and adaptive immune system have been shown to be perturbed in SCD. For example, invariant natural killer T (iNKT) cells have been shown to be more numerous and activated and promote pulmonary inflammation and dysfunction in SCD, and their depletion can lead to clinical improvement⁶⁻⁸. Additionally we have previously shown that lymphocyte populations are globally elevated in patients with SCD, particularly in patients who are not treated with hydroxyurea⁹. This may have important implications in patients undergoing reduced intensity or nonmyeloablative hematopoietic stem cell transplantation as higher lymphocyte numbers in transplant recipients may contribute to graft rejection.

Natural killer cells (NK cells) are lymphocytes that were named for their innate ability to lyse leukemia and lymphoma cells. The ability of NK cells to kill has been correlated with their phenotype based on surface expression of markers of maturity and activation. Thus, CD56^{bright}-expressing NK cells are considered immature NK cells and in response to exposure to a target produce high levels of interferon-gamma, but have limited killing capacity¹⁰, while mature CD56^{dim} NK cells, on the other hand, demonstrate higher levels of cytotoxicity¹¹. Similarly, mature NK cells are characterized by expression of CD57 and killer immunoglobulin-like receptors (KIR)¹². Exposing NK cells to a stimulus such as interleukin-2 leads to increased expression of markers associated with activation such as the natural cytotoxicity receptors (NKp30, NKp44, NKp46) and NKG2D making these markers useful to identify NK cells that are in a stimulatory environment¹³.

Graft rejection has limited the success of hematopoietic stem cell transplantation to cure patients with SCD¹⁴⁻¹⁶. This problem is likely to become more important as modern transplant approaches for SCD decrease the intensity of pre-transplant conditioning to limit secondary side effects. Such reduced intensity transplant regimens result in the persistence of SCD recipient immune cells that may contribute to graft rejection. NK cells are capable of graft rejection in the absence of T cell activity^{17,18}. If the NK cells in patients with SCD have increased function it is conceivable that these cells may more readily kill donor hematopoietic stem cells. Hence, normalizing NK cell function prior to transplantation using disease modifying therapy may decrease the risk of rejection.

We have previously shown that the absolute number of NK cells is higher in patients with SCD not on disease modifying therapy⁹. In the current study, we hypothesized that patients with SCD not on disease modifying therapy have increased cytolytic function and a higher

number of NK cells with mature phenotype and expression of markers associated with activation compared to healthy controls.

Methods

Study participants:

Peripheral blood from patients (age 1 to 21 years) with SCD (HbSS/HbS β^0 thalassemia) was collected from patients in steady state who had no acute SCD complication, fever or infection in the preceding four weeks. Control blood was obtained from age- and race-matched healthy controls. Patients were categorized based on treatment with hydroxyurea (minimum six weeks on therapy), chronic red blood cell transfusion, or no disease-modifying therapy (no hydroxyurea or transfusions). Patients on combination therapy with hydroxyurea and transfusion therapy were excluded. Control subjects and SCD patients who were receiving other treatments or systemic therapies that may have affected NK cell function were excluded from participating in this study.. Research participants were consented and assented under Institutional review board approved protocols at Children's National Health System and in accordance with the declaration of Helsinki.

Immunophenotyping of NK cells:

From whole peripheral blood, one million white blood cells were stained and dead cells were excluded using LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit (Life Technologies). Red blood cells were lysed using ACK lysing buffer (Quality Biological) per manufacturer recommendations. NK cell counts were calculated using the percentage of CD3⁺CD56⁺ and absolute lymphocyte count from the patient's CBC. NK cells were further characterized using the following antibodies from Miltenyi Biotech except where otherwise specified : CD3 APC-Vio770, CD56 PerCP-Vio700, CD57 VioBlue, CD336 (NKp44) VioBright FITC, CD337 (NKp30) Phycoerythrin (PE), human, CD314 (NKG2D) PE-Vio700, CD69-allophycocyanin (APC) human, CD158a (KIR2DL1)-APC, CD158b PE (BD Bioscience), CD158b2 (KIR2DL3)- fluorescein isothiocyanate (FITC), CD158e (KIR3DL1) PE-Vio770, CD45 Vioblue. Isotype controls were obtained from Miltenyi and BD Bioscience. Events were acquired on a MACSQuant analyzer (Miltenyi Biotech) and analyzed using FlowJo software (TreeStar). A minimum of 10,000 viable lymphocyte events were collected per sample.

NK cell degranulation assay:

Within 24 hours of blood collection and without cryopreservation, peripheral blood mononuclear cells (PBMCs) were separated by Ficoll density gradient and co-incubated with K562 cells (ATCC, Cat no. CCL243) at 37 Degrees for 4 hours in RPMI-1640 media supplemented with 10% FBS at a PBMC:K562 ratio of 10:1. Golgi plug (brefeldin A) and Golgi stop (monensin) reagents (BD BioScience) were added at the start of the incubation per manufacturer instructions. The following antibodies were also added CD3 PE-Cy5, CD56 PE, CD107a APC (Miltenyi). After incubation, cells were fixed and permeabilized using the Cytofix/Cytoperm Plus Kit (BD BioScience) and intracellular staining performed with anti-IFN-gamma FITC (Miltenyi). A minimum of 200,000 events were acquired by flow cytometry.

Cytotoxicity assay:

The cytotoxicity of NK cells was determined by measuring the ability to kill immortalized cells from a leukemia cell line (K562 cells) that is commonly used to assess NK cell function in vitro. This assay was performed in patients with sufficient blood collection and an adequate number of NK cells following magnetic cell selection (650,000 NK cells). Fresh PBMCs underwent selection with an NK cell selection kit (Miltenyi, Cat No. 130-092-657) and purity of CD3-CD56⁺ cells determined by flow cytometry. Based on CD3-CD56⁺ percentage, NK cells were plated at varying effector to target ratios (10:1, 5:1, 2.5:1, 1.25:1) with K562 cell targets labeled with CellVue® Claret (Sigma Cat# MIDCLARET-1kt) to allow discrimination. Cells co-incubated in RPMI-1640 media supplemented with 10% FBS 4 hours at 37 degrees Celsius. At the end of the co-incubation, the dead cell marker 7-amino-Actinomycin D (7AAD, BD Bioscience) was added and the percentage of dead K562 cell was quantified by flow cytometry acquisition of at least 20,000 target cells. The NK cell line, NK92 MI, (ATCC, Cat no. CRL-2408) was used as reference sample in each assay and patient sample results were standardized to this reference as previously described¹⁹. Cytotoxicity was calculated as follows: % of specific cell death = $100 \times (\% \text{Dead target cells} - \% \text{Spontaneous target cell death}) \div (100 - \% \text{Spontaneous target cell death})$.

Statistical analysis:

Non-parametric testing was used to compare the demographic, NK phenotypic and degranulation data (Kruskal-Wallis test and where significant differences detected Dunn's multiple comparison test performed). Fisher's exact test was used for categorical demographic variable comparison. For cytotoxicity analyses, a two-way ANOVA was performed followed by Dunnett's comparison test. Pearson's correlation coefficient was used to assess correlation between clinical parameters and absolute NK cell number. All analyses and figures were generated in Graphpad PRISM 7.02.

Results

Demographics

Demographics of healthy controls and patients are shown in Table 1. Patient age and gender were comparable amongst all groups. As expected, there were higher total white blood cell, neutrophil and lymphocyte counts observed in the patients with SCD compared to healthy controls. The median hydroxyurea dose was 24.4 mg/kg/day (range 13.7–30.6) and mean HbS% in the chronic transfusion group measured prior to transfusion was 33.3% (range 16.6–60.7). The mean corpuscular volume was highest in the hydroxyurea group as expected.

Increased number of activated and mature NK cells in patients with SCD not on hydroxyurea or transfusion

In agreement with previous reports, absolute NK cell number, defined as the absolute CD3-CD56⁺ lymphocyte population (Fig. 1A), was significantly higher in patients who were not receiving disease modifying therapy compared to healthy controls and patients on hydroxyurea (Fig. 1B). The difference in absolute NK cell number between the transfusion

and other groups did not reach statistical significance (no disease modifying therapy vs. transfusion, $p = 0.21$; controls vs. transfusion, $p > 0.99$; hydroxyurea vs. transfusion, $p > 0.99$). Additionally patients not on disease modifying therapy had higher NKG2D+, NKp30+, CD56dim+, CD57+ and KIR+ cells compared to healthy controls (Fig 1C, 1D, 1G, 1H and 1I) but there were not significant differences in NKp44 and CD69 (Fig. 1E and 1F). Interestingly, patients on hydroxyurea had lower NKG2D+, NKp30+, CD56dim+, and KIR+ NK cells compared to patients not on disease modifying therapy (1C, 1D, 1G and 1I), and similar cell counts compared to healthy controls for all markers (1C-1I), suggesting that hydroxyurea could normalize the NK cell phenotype in patients with SCD. There was a statistically significant increase in the number of NKp44+ NK cells in the transfusion group compared to the healthy control group (Fig 1E) but this was the only difference seen comparing the transfusion group to controls, no disease modifying therapy and hydroxyurea groups.

Absolute number of NK cells is not associated with hemoglobin level or reticulocyte count but is associated with hemoglobin F (HbF) percentage

We next analyzed the group of patients with SCD that included both those not on disease modifying therapy and those on hydroxyurea, and found that in this group the number of absolute NK cells appeared to be inversely associated with HbF percentage (Pearson correlation coefficient $r = -0.51$, $p = 0.044$) (Fig. 2A). There was no correlation seen with absolute NK cell number and either hemoglobin level (Fig. 2B) or reticulocyte count (Fig. 2C).

NK cells from patients with SCD not on disease modifying therapy have increased cytolytic activity compared to healthy controls and patients on disease modifying therapy

We next determined if the NK cells in the SCD cohort had different functional characteristics compared to healthy controls. We assessed NK cell function in two different ways, 1) a degranulation and activation assay that measured NK cell degranulation (NK cell surface CD107a expression) and activation (intracellular IFN-gamma expression) following co-incubation of NK cells with a target (K562 cells) (Fig. 3), and 2) A cytotoxicity assay that measured the ability of the NK cells to lyse K562 cells (Fig. 4).

The ratio of the mean fluorescence intensity (MFI) of NK cells co-incubated with K562 to unstimulated NK cells from the same patient were calculated for both CD107a and IFN-gamma expression. The degranulation (CD107a) in the control and no disease modifying therapy groups were similar to the hydroxyurea group having lower expression though this difference was not statistically significant ($p = 0.705$, Fig. 3B). There was a trend to higher NK cell activation (IFN-gamma expression) in the no disease modifying therapy group compared to the healthy control and hydroxyurea groups ($p = 0.097$, Fig. 3C).

Next, the cytolytic function of NK cells was evaluated in select patients and controls who had sufficient blood collected to perform cytotoxicity assays (minimum of 650,000 NK cells). NK cells from SCD patients not on disease modifying therapy ($n = 8$) demonstrated a significant increased killing of K562 cells compared to healthy controls ($n = 5$) while NK cell

cytotoxicity in patients on therapy with hydroxyurea or chronic transfusion (n=3) were not significantly different from healthy controls (Fig. 4).

Discussion

There is mounting evidence that SCD is not only a disorder characterized by red blood cell pathology but is also associated with inflammation^{1-5,7,9,20}. The current study of a new cohort of patients from a different location supports our previous finding that children with SCD who are not on disease modifying therapy with hydroxyurea or chronic red cell transfusions have higher numbers of NK cells in their peripheral blood⁹. In this study we expanded on this finding by characterizing the NK cell phenotype and function which had not been previously done. As studies of other immune cells in patients with SCD have pointed to increased immune cell numbers and activated phenotype, we investigated if a similar phenomenon was also occurring in the NK cells of these patients.

To do this, we selected cell surface markers previously shown to be associated with NK cell activation (NKG2D, NKp30, NKp44, CD69) and maturity (CD57+, CD56dim+, KIR+) due to their association with increased cytotoxic function¹¹⁻¹³. A strength of our study was that we assessed NK cell phenotype and function within 24 hours of sample collection and avoided cryopreservation and culturing with cytokines as these have been shown to alter NK cell function^{19,21}. We believe this assessment of fresh NK cells best reflects their activity in vivo. In keeping with our hypothesis that patients not on disease modifying therapy have more activated NK cells, we observed the patients on therapy (primarily hydroxyurea) had similar numbers of NK cell subpopulations compared to healthy controls, whereas those not on therapy had higher numbers of NK cells with markers associated with maturity and activation. From a functional perspective, we observed a trend to decrease in NK cell interferon gamma production in response to a stimulus (K562 cells) in patients on hydroxyurea therapy compared to those not on disease modifying therapy. However we acknowledge that this is likely an underrepresentation of the overall Th1 cytokine response since we only evaluated a single Th1 cytokine. More importantly and of potential clinical significance we observed increased NK cell cytolytic activity in patients not on disease modifying therapy. Hence, this data supports the notion that disease modifying therapy such as hydroxyurea can normalize the increased NK activation/cytotoxicity and function seen in patients with sickle cell disease.

Fetal hemoglobin (HbF) production is increased by hydroxyurea and has anti-sickling activity^{22,23}. Less sickling should result in less hemolysis and inflammation. In support of this concept we found that HbF was inversely proportional to absolute NK cell number when analyzing patients not on chronic transfusion. While the mechanism by which hydroxyurea normalizes NK cell number and function remains unknown, our findings suggest that there may be an association between HbF induction and normalization of NK cell numbers which may have implications for patients as they proceed to hematopoietic stem cell transplantation. Many patients with SCD who undergo transplant are treated with chronic transfusions pre-transplant. Traditionally, concomitant hydroxyurea treatment is not recommended with chronic transfusions. It is worth noting that Dedeken et al found a significant decrease in the risk of graft rejection after all patients with SCD undergoing

transplant (including patients who had been on chronic transfusion) were routinely treated with hydroxyurea in the pre-transplant period²⁴. Our finding that hydroxyurea appears to normalize the increase NK cytotoxicity may have implications for patients who will undergo hematopoietic stem cell transplantation. Especially in the context of reduced intensity or nonmyeloablative transplant, host immune cells including NK cells may not be completely eradicated and could potentially contribute to graft rejection²⁵. This is also of interest in the HLA-mismatched transplant setting where graft rejection continues to be a problem¹⁵. Further study is required to determine the impact of host NK cells on graft rejection in transplant for SCD.

Our study is limited by its cross sectional nature. Study of patients before and after the implementation of disease modifying therapy is needed to more definitively establish how treatment with hydroxyurea or chronic transfusion affects NK cells. There were no significant differences in the absolute neutrophil counts and hemoglobin levels between the “no disease modifying therapy” group and the “hydroxyurea” group. Since hydroxyurea is well known to decrease neutrophil count and increase hemoglobin in patients with SCD, the lack of these expected differences in these groups likely reflects confounding by indication. Patients with lower baseline hemoglobin (and higher baseline absolute neutrophil count) were more likely to be treated with hydroxyurea which then normalized these values. It is therefore likely that patients with SCD who had less severe complications were not on hydroxyurea and as a result, had higher baseline hemoglobin levels and lower absolute neutrophil counts. However our study adds important phenotypic and functional data on NK cells in children with SCD and will be valuable to those studying hematopoietic stem cell transplantation and rejection in this population. In addition, the impact of NK cells on SCD pathology would be important to study as mouse models of SCD suggest NK cells in addition to iNKT cells may play a role in pulmonary inflammation²⁶. Future work will focus on longitudinal studies of NK cell function in patients who begin hydroxyurea in combination with chronic transfusion and also in patients who receive hematopoietic stem cell transplantation to determine if NK cell function impacts graft rejection.

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Abbreviation

7-AAD	7-aminoactinomycin
ACK	Ammonium-Chloride-Potassium
APC	allophycocyanin
ATCC	American Type Culture Collection
CBC	Complete blood count

CD	Cluster of Differentiation
FITC	Fluorescein isothiocyanate
Hb	Hemoglobin
HLA	Human leukocyte antigen
IFN	Interferon
iNKT	invariant natural killer T
KIR	Killer-like immunoglobulin receptor
NK	Natural Killer
PE	Phycoerythrin
PBMC	peripheral blood mononuclear cell
RPMI	Roswell Park Memorial Institute
SCD	Sickle Cell Disease

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Figure 1. Comparison of absolute NK cell numbers in peripheral blood of patients with sickle cell disease based on treatment category and healthy controls.

Absolute NK cell numbers were quantified (A) and further characterized by phenotype: markers associated with activation (B-E) and maturity (F-H). Mean and standard error of the mean (SEM) are displayed. Kruskal-Wallis test *** P ranges >0.0001 to <0.001 , ** P ranges >0.001 to <0.01 , * P ranges >0.01 to <0.05 .

Figure 2. Correlation between clinical laboratory parameters and absolute NK cell count in patients with sickle cell disease including those not on disease modifying therapy and those on hydroxyurea.

Linear regression lines and Pearson's correlation coefficients, r , of hemoglobin F (HbF) percentage (A), hemoglobin (B) and absolute reticulocyte count (C) on day of NK cell assessment in patients on supportive care or hydroxyurea are shown.

Figure 3. Assessment of NK cell degranulation and interferon-gamma (IFN-gamma) production in patients with sickle cell disease.

Shown is a representative plot of a patient with sickle cell disease not on disease modifying therapy (A). NK cells were co-incubated with K562 cells and CD107a surface expression as a surrogate for degranulation (B) and intracellular interferon-gamma (C) measured in response to the stimulus. Mean and standard error of the mean (SEM) are displayed in (B) and (C). Kruskal-Wallis test *** P ranges >0.0001 to <0.001, ** P ranges >0.001 to <0.01, * P ranges >0.01 to <0.05.

Figure 4. Comparison of NK cell cytotoxicity in patients with sickle cell disease not on disease modifying therapy with healthy controls.

NK cell cytotoxicity was determined at different effector to target ratios (10:1, 5:1, 2.5:1, 1.25:1) for each group and standardized to the cytotoxicity of the reference sample NK cell line NK92 MI. A statistically significant difference between patients in the no disease modifying therapy group (n=8) and healthy controls (n=5) is denoted (**). No difference was seen between those on therapy (hydroxyurea or transfusion, n=3) and the control group. Mean and standard error of the mean (SEM) are displayed. Ordinary two-way ANOVA with Turkey's multiple comparison test. *** P ranges >0.0001 to <0.001, ** P ranges >0.001 to <0.01, * P ranges >0.01 to <0.05.

TABLE 1.

Demographic and cell counts by treatment group

	Healthy controls (n=23)	Sickle Cell Disease (n=44)			
		No Disease-Modifying Therapy (n=20)	Hydroxyurea (n =13)	Chronic Transfusion (n = 11)	p-value
Age, years, median, (range)	13 (1–20)	7 (2–16)	11 (2–18)	8 (4–20)	0.54
Male, %	43.5	55	38.4	72.7	0.32
WBC, $\times 10^3/\text{ul}$, median, (range)	6.15 (3.62–9.59)	11.18 (5.48–25.31)	9.51 (4.51–15.65)	12.68 (5.08–19.24)	<0.0001
ANC, $\times 10^3/\text{ul}$, median, (range)	2.21 (1.23–5.27)	4.84 (2.17–16.96)	4.25 (1.27–9.23)	7.29 (1.91–10.74)	<0.0001
ALC, $\times 10^3/\text{ul}$, median, (range)	2.51 (1.38–6.6)	4.33 (2.18–7.14)	4.44 (2.09–6.24)	3.91 (2.29–6.56)	0.01
Hgb, gm/dL, median, (range)		8.5 (6.9–10.5)	8.6 (6.9–11)	8.9 (7.3–11.5)	0.6
MCV, fL, median, (range)		77.45 (66.2–91.2)	92.6 (75.4–106.9)	85.4 (73.3–88.1)	<0.0001
ARC, median, (range)		296 (154–503)	335 (125–539)	401 (214–771)	0.35
Hb F, %, median, (range)		17.1 (0.5–26.6)	15.5 (5.0–31.5)	1.3 (0–4.1)	0.0006

Comparisons were performed using the Kruskal Wallis test except for gender where Fisher's exact test was used. P-values <0.05 were considered significant. WBC = White blood count, ANC = Absolute neutrophil count, ALC = Absolute lymphocyte count, Hgb = Hemoglobin level, ARC = absolute reticulocyte count, HbF = Hemoglobin F, MCV = Mean Corpuscular Volume