

**Evaluation of purified Natural Killer cell- functions in  
Familial Hemophagocytic Lymphohistiocytosis.**

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## Evaluation of purified Natural Killer cell- functions in Familial Hemophagocytic Lymphohistiocytosis.

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**Abstract**

**Background:** Familial Hemophagocytic lymphohistiocytosis (FHL) is a rare, genetic, immune dysregulation disorder of aberrant hyperactivation of lymphocytes causing inflammation and hemophagocytosis. We report a three month old male who was evaluated for the possibility of FHL due to a positive family history. The patient was asymptomatic, however, the soluble Interleukin 2 (IL-2) receptor was elevated and Natural Killer (NK) cells quantity and function were severely decreased.

**Methods:** Purification of NK cells and evaluation of NK cells cytotoxicity and IFN $\gamma$ /TNF $\alpha$  secretion after IL-2 activation relative to the patient family members.

**Results:** The patient's NK specific lysis was enhanced compared to his mother and slightly higher than his sister. The IFN $\gamma$  and TNF $\alpha$  secretion by the patient's NK cells after challenge with target 721 cells or anti-Natural Cytotoxicity Receptors (NKp30 and NKp44) antibodies showed levels that are close to mother's and sister's NK secretion levels. Due to low yield of NK cells from patient's father the results for his NK cells are incomplete.

The patient did not undergo HSCT and continued to be followed. He is now seven years old and thriving without signs of FHL. His last examination was in August 2012 for functionality of isolated NK cells. The results showed normal cytotoxicity, cytokine secretion and CD107a up-regulation to the NK cell surface (data not shown).

**Conclusion:** We propose that NK function assessment in patients with presumed FHL should be performed on isolated NK cells populations.

This practice may reduce false-negative results in NK function assays.

**Statement of Novelty:** In this case report we show that functional assessment of unpurified NK cells could lead to false-negative assessment in one of the parameters in FHL. Assessment of NK function without NK purification may lead to an erroneous diagnosis of poor NK function.

**Keywords:** Familial Hemophagocytic lymphohistiocytosis (FHL), soluble Interleukin 2 Receptor (sIL2R), hematopoietic stem cell transplantation (HSCT), Natural Killer (NK), NK cytotoxicity assay.

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## **Introduction**

Familial haemophagocytic lymphohistiocytosis (FHL) is a rare autosomal recessive disorder with an incidence of 0.12 per 100,000 children per year. FHL characterized by persistent hyperproliferation, hyperactivation and infiltration of histiocytic and T-lymphocyte populations due to systemic hypercytokinemia and defective lymphocytic cell cytotoxicity [1–4]. The diagnosis of FHL is suggested by criteria including: prolonged fever, cytopenia, splenomegaly, hypertriglicidemia or hypofibrinogenemia, hemophagocytosis, hyperferritinemia, and elevated soluble Interleukin-2 receptor (sIL2R) blood levels [5–8]. Currently, 4 specific genetic defects are recognized, causing most of FHL cases and they are associated with CTL and NK cell cytotoxicity [2,9]. Consequently, one of the most important pathogenic mechanism in FHL patients is the impaired or absent Natural Killer (NK) and T-cell cytotoxicity regardless of normal quantity of cells [2,3,6,9–11].

The main treatment for FHL is hematopoietic stem cell transplantation (HSCT) that improves the prognosis of this otherwise fatal disease [2,5,6,12]. The success of HSCT is partly determined by having active disease at the time of HSCT [8,13], therefore it is crucial to make an early diagnosis of FHL. The diagnosis of FHL may be difficult since laboratory evaluation may be normal in patients with FHL shortly before clinical signs appear [8,14].

We present a child who was suspected of having FHL due to a positive family history with severely reduced NK cell quantity, leading to an erroneous result of reduced NK cell function.

## **Materials and Methods**

The patient was evaluated at 2 months of age for FHL due to a family history. Five siblings died within the first year of life due to a severe hyper-inflammatory disease that occurred after an infection in the first weeks/months of life. In one of the siblings a diagnosis of FHL was considered with partial fulfillment of the diagnostic criteria for FHL, the evaluation was not completed since we decided to refer the patient to hematopoietic stem cell transplant based on clinically based suspicion. Genetic evaluation in this sibling showed absence of mutations in the perforin gene. No other genetic tests were available at that time. We therefore actively evaluated the patient for signs of FHL since birth. The parents are healthy first cousins of Arab Bedouin ethnicity. The patient was asymptomatic,

physical examination was unremarkable, without hepatosplenomegaly or rash. The CBC, cholesterol and triglycerides levels were normal, ferritin level was normal, however, the sIL2R was elevated at 3638 U/ml (normal 300-2000 U/ml). This abnormality taken together with the family history prompted the performance of an NK quantity and function assay. At three months of age low NK (CD56+) number of  $0.06 \times 10^3$  cells/ $\mu$ L (normal  $0.3$ – $0.7 \times 10^3$  cells/ $\mu$ L) was measured, constituting 1.9% of total lymphocytes (CD45+ gated). The rest of the lymphocytes subpopulation numbers were normal. Similar results were recorded at the age of 7 months, NK count was  $0.11 \times 10^3$  cells/ $\mu$ L, constituting 1.4% of total lymphocytes.

Initial, cytotoxicity functional assay of NK cells was performed at 3 months of age, using peripheral blood mono-nuclear cells (PBMCs) at ratio 50:1 Effector: Target K562 cells, showed no activity (0% of specific lysis) whereas control healthy donors showed normal cytotoxicity (57.1% or 61.8% specific lysis). The amount of NK cells were still low but slightly improved 4 months after the initial evaluation:  $0.11 \times 10^3$  cell/  $\mu$ L (normal  $0.3$ – $0.7 \times 10^3$  cells/ $\mu$ L).

The patient remained asymptomatic and was followed up with two positive criteria for FHL. Since we were hesitant to suggest a pre-emptive HSCT based solely on family history and these positive laboratory criteria for FHL (elevated sIL2R, NK cytopenia, and no NK cytotoxicity function), a further analysis of NK function was performed.

NK cells were isolated from the patient's peripheral blood, as well as from his parents and his healthy 4 year old sister. In contrast to the routine lysis assay that uses PBMCs for the cytotoxicity assay, the target cells were mixed with counted number of purified NK cells. The NK cells from the isolated fraction were counted by flow cytometry (CD56+, CD3-). In addition to the isolation, the cells were pre-activated over night with 300 Units/ml of Interleukin 2 (IL-2). NK cells isolation, cytotoxicity and cytokine secretion assays were performed as previously described [15,16].

This study was approved by the Soroka University Medical Center ethical committee.

## **Results**

In the cytotoxicity assay the patient's NK cells lysed the target cells (Fig.1A). Additionally, the patient's NK specific lysis was enhanced compared to his mother and slightly higher than his sister (Fig.1A). The IFN $\gamma$  and TNF $\alpha$  secretion by the patient's NK cells after challenge with target 721 cells or anti-Natural Cytotoxicity Receptors (NKp30

and NKp44) antibodies showed levels that are close to mother's and sister's NK secretion levels (Fig.1B-C). Due to low yield of NK cells from patient's father the results for his NK cells are incomplete.

The patient did not undergo HSCT and continued to be followed. He is now seven years old and thriving without signs of FHL. His last examination was in August 2012 for functionality of isolated NK cells. The results showed normal cytotoxicity, cytokine secretion and CD107a up-regulation to the NK cell surface (data not shown).

### **Discussion**

We present a case of an asymptomatic child with presumed FHL based on a positive family history and 2 diagnostic criteria (elevated sIL2R and decreased NK quantity and function). The initial NK function assessment did not take into account the extremely low levels of NK cells in peripheral blood and was considered to be without any NK cytotoxicity. Our extended workup showed that purified NK cell possessed normal cytotoxic activity. Therefore the very low NK numbers hindered the NK cytotoxicity assay but purification of the NK cells, prior to the functional test, allowed differentiation between the number of NK cells and their function. Decreased function and quantity of NK cells are one of the most important criteria for the diagnosis of FHL, and discounting the NK fraction when assessing NK function may cause an erroneous result, giving a false negative NK function assay.

Cytotoxic function of human cord-blood derived NK cells was shown to be lower than that of adult NK cells. Activation of these cord-blood NK cells and adult NK cells with IL-2 resulted in similar cytotoxic activity of cord blood NK cells to adult NK cells [17]. The first routine lysis assay did not include IL-2 activation whereas the extended analysis included overnight IL-2 incubation. Therefore, this suggests that a complete NK cell assessment from very young infants or neonates should include activation with IL-2.

The diagnostic criteria for FHL are not specific, viral infections or malignancies in children without FHL can cause prolonged fever, cytopenia, hepatosplenomegaly, increased ferritin, and hemophagocytosis [3,4,18]. Our patient did not have FHL and yet with a positive family history and two criteria for FHL the diagnosis was not unlikely. Thus there is a large differential diagnosis of clinical presentations similar to FHL and this diagnosis should be done very carefully.

Since the previous 5 siblings became ill in the first months of life and succumbed in the first year, we can now safely assume on clinical grounds alone that our patient does not have the same disease and is not predisposed to developing FHL. The normal NK function also contributes to this assumption.

Our case report suggests that NK assessment should take into account the number of NK cells in peripheral blood. Either NK cell purification or correction for NK quantity should be considered when assessing NK function in peripheral blood and purification of NK cells allows assessment of NK function even when very few NK cells are present. Activation of NK cells with IL-2 can enhance the credibility of NK cell function assessment in neonates and very young infants.

### **Conflicts of interest**

The authors declare that they have no conflict of interest.

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## **Figure Legend**

### **Figure 1. Cytotoxicity and cytokine secretion by isolated human NK cells.**

(A) Target 721.221 cells were radioactively labeled with  $S^{35}$  for 12 hrs, washed and plated ( $5 \times 10^4$  target cells/ well) in 96 U-shaped plate and incubated with primary NK effector cells and NK-92 NK cell line as control in the indicated effector to target cells ratio (the experiment was performed in four-plicate). The lysis of targets by NK cells was assayed in a standard 5-h  $S^{35}$  release assay. (B-C) NK effector cells ( $2 \times 10^4$  cells/ well) were incubated with 721.221 target cells, pre-coated with mAb against NKp30 or NKp44 wells, or without activation for 18 hrs (the experiment was performed in four-plicate). For father's NK cells only anti NKp30 activation mono-plicate was performed, for the sister's NK cells anti NKp44 and 1:1 effector to target ratio were not performed. (B) ELISA O.D. of relative  $IFN\gamma$  levels in the culture supernatants are shown. (C) ELISA O.D. of relative  $TNF\alpha$  levels in the culture supernatants are shown. Bars  $\pm$  SD.

