Analysis of Natural killer cell function in familial hemophagocytic lymphohistiocytosis (FHL). Defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease.

Running title: NK cells and CD107a expression in FHL

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Abstract

Natural killer (NK) cells from patients with familial hemophagocytic lymphohistiocytosis due to PRF1 (FHL2, n=5) or Munc13-4 (FHL3, n=8) mutations were cultured in IL-2 prior to their use in various functional assays. Here we report on the surface CD107a expression as a novel rapid tool for identification of patients with Munc13-4 defect. Upon target interaction and degranulation, FHL3 NK cells displayed low levels of surface CD107a staining, in contrast to normal controls or perforin-deficient NK cells.

B-EBV cell lines and dendritic cell targets reveal the FHL3 NK cell defect while highly susceptible tumor targets were partially lysed by FHL3 NK cells expressing only trace amounts of Munc13-4 protein. Perforin-deficient NK cells were completely devoid of any ability to lyse target cells. Cytokine production induced by mAb-crosslinking of triggering receptors, was comparable in patients and normal controls. However, when cytokine production was induced by co-culture with 721.221 B-EBV cells, FHL NK cells resulted high producers, whereas control cells were almost ineffective. This could reflect survival versus elimination of B-EBV cells i.e. the source of NK cell stimulation, in patients vs healthy controls, thus mimicking the pathophysiological scenario of FHL.
Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare, heterogeneous fatal disease of early infancy characterized by an hyperinflammatory syndrome with fever, hepatosplenomegaly, cytopenia, hypertriglyceridemia, hypofibrinogenemia and, in some cases, central nervous system (CNS) alteration. Histological examination of involved organs (more commonly bone marrow aspiration) typically shows infiltration of lymphocytes and histiocytes with hemophagocytosis. Characteristic findings are also the high levels of various cytokines, such as interleukin (IL)-6, IL-8, IL-10, IL-18, interferon (IFN)-γ and tumor necrosis factor (TNF)-α and also high plasma concentrations of sCD25 and sCD95-ligand. In some cases HLH may occur in patients of any age undergoing therapeutic immune suppression. In such cases, also defined as "secondary", immune suppressive treatment withdrawal may result in control of HLH. The remaining (also called "primary") forms are of genetic origin and often defined as familial hemophagocytic lymphohistiocytosis (FHL). A genetic heterogeneity underlies FHL. The most common genetic defects in FHL patients involve the perforin gene (PRF1) on chromosome 10q21 and the Munc13-4 gene on chromosome 17q25. Mutations of PRF1 account for approximately 30-40% of patients, defined as FHL2 subtype, and Munc13-4 mutations are identified in an additional 25-30% of cases (FHL3). The proteins encoded by both genes are implicated in the killing machinery. Very recently, mutations in the syntaxin11 gene (6q24) have been reported in a small group of patients (FHL4) with common Kurdish origin. This defect is thought to alter intracellular vesicle trafficking of the phagocytic system.

*PRF1* gene encodes perforin as an inactive precursor form, which, after processing in a post-Golgi apparatus by proteolysis and glycosylation, becomes an active protein. This mature form of perforin is stored with granzymes in specialized secretory lysosomes, known as lytic granules, which are present in Natural Killer (NK) and cytotoxic T lymphocytes (CTL). Upon target cell interaction, lytic granules polarize and release their content at the immunological synapse. The secreted perforin then inserts into the lipid bi-layer and following polymerization generates poly-perforin pores in the plasma membrane of target cells. This pore formation leads to the destruction of cells by osmotic lysis and by allowing entry of
apoptosis-inducing granzymes. Munc13-4, a member of Munc13 family of proteins involved in vesicle priming function, has been described as a positive regulator of secretory lysosome exocytosis. While Munc13-1 functions as a priming factor in neural cells, Munc13-4 is highly expressed in several hematopoietic cells. In FHL3 patients, Munc13-4 deficiency results in defective cytolytic granule exocytosis, despite polarization of the lytic granules and docking with the plasma membrane. Recent evidence shows that Munc13-4 binds to Rab27A; the two proteins colocalize on the membranes of secretory lysosomes in CTL and mast cells and promote the dense core granule secretion in platelets. Rab27A is highly expressed in melanocytes, hematopoietic and other secretory cells. Absence of functional Rab27A causes the Griscelli syndrome type 2 (GS2), a genetic disorder characterized by defects of pigmentation and of granule exocytosis in CTL, in which lytic granules fail to dock on the plasma membrane and therefore do not release their content. Thus, these fatal genetic disorders which display similar pathologic and clinical features all disrupt the release or function of cytotoxic proteins.

Defects in cellular cytotoxicity, excessive production of inflammatory cytokines and abnormal macrophage activation characterize HLH. In these patients an impairment of cytolytic activity of NK cells, that provide the first line of host defence, and subsequently of CTL, results in a markedly reduced ability to control viral infection. Uncontrolled viral dissemination together with a parallel excessive inflammatory reaction results in extensive tissue damage. Two major mechanisms of cytotoxicity are perforin/granzyme- and death receptor (e.g. FasL and TRAIL)-mediated pathways. Many studies, including experiments with perforin-deficient mice, led to the conclusion that NK cells primarily use the perforin/granzyme-pathway to eliminate virus-infected or transformed cells. Function of NK cells is regulated by an array of different receptors. Human NK cells are equipped with activating receptors (i.e. the NCRs NKp46, NKp30 and NKp44, NKG2D, DNAM-1) and coreceptors (i.e. 2B4, NKp80, NTBA and CD59) that once engaged by the specific ligands on target cells induce their lysis. The function of activating receptors is under the control of inhibitory NK receptors, namely KIRs (CD158), which recognize shared allelic determinants of classical HLA-A, -B or -C, and the CD94/NKG2A heterodimeric receptor, which interacts with HLA-E. The general concept is that MHC-class I-deficient aberrant cells are susceptible to NK-mediated lysis while normal cells are protected from NK cells by the expression of HLA class I
molecules.\textsuperscript{35} However, among normal cells an exception is represented by immature DC (iDC), which are characterized by low amounts of surface HLA class I molecules, particularly HLA-E, which render them highly susceptible to lysis by autologous NK cells.\textsuperscript{36} In contrast, mDC, expressing higher levels of HLA class I molecules, are protected from NK-cytotoxicity in an autologous setting. Importantly, NK cells are capable of negatively selecting those DC that did not acquire the capability of optimal Ag presentation and T cell priming\textsuperscript{37-40}. It is of note that, in response to virus-infected or tumor-transformed cells, NK cells also release cytokines and chemokines which can activate and/or recruit multiple cell types, thus contributing to the inflammatory response.\textsuperscript{41,42}

Differential diagnosis of HLH may be difficult.\textsuperscript{43} The presence of a family history of HLH-like episodes, or consanguinity, may induce the suspicion of a genetic defect underlying HLH. Yet, analysis of genetic mutations is not widely accessible, as time consuming and expensive. Recently, cytofluorometric analysis of perforin expression became available,\textsuperscript{44} while rapid screening of FHL3 has not been reported so far.

In the present study, we characterized the functional patterns of NK cells derived from patients with the two most frequent subtypes of FHL (FHL 2 and 3). Our data also provide a novel tool for rapid identification of FHL3 patients and discrimination between genetic defects.
Materials and Methods

Patients
This study was approved by the institutional review board at the Istituto G. Gaslini. Peripheral blood samples were obtained from patients with HLH, diagnosed following current diagnostic criteria, after informed consent according to the Declaration of Helsinki. Five FHL2 patients were included in this study and the mutations in PRF1 gene are listed in Table I. Eight FHL3 patients with Munc13-4 gene mutations are described in Table II. Main clinical features of these patients are summarized in Table III. All patients were treated according to HLH-94 protocol.

PRF1 and Munc13-4 Genes Sequencing
Sequences of PRF1 and Munc13-4 genes were retrieved from the National Center for Biotechnology Information (NCBI). To analyze PRF1 and Munc13-4 genes, exons and adjacent intronic regions were amplified, from genomic DNA, and directly sequenced in both directions (BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA). Sequence primers used for amplification are available on request. Sequences obtained by ABI PRISM 3130 Sequence Detection System (Applied Biosystem) were analyzed and compared to the reported gene structure using the dedicated software SeqScape (Applied Biosystem).

Monoclonal antibodies and cytofluorometric analysis
The following mAb, produced in our laboratory, were used in this study: JT3A (IgG2a, anti-CD3), c127 (IgG1, anti-CD16), c218 (IgG1, anti-CD56), BAB281 and KL247 (IgG1 and IgM, respectively, anti-NKp46), Z231 and KS38 (IgG1 and IgM, respectively, anti-NKp44), A76 and F252 (IgG1 and IgM, respectively, anti-NKp30), BAT221 (IgG1, anti-NKG2D), MAR206 (IgG1, anti-CD2), PP35 (IgG1, anti-2B4) and A6-136 (IgM, anti-HLA class I). Anti-CD56-PC5 (N901, IgG1 mAb, Beckman Coulter, Marseille, France), anti-CD3-FITC (HIT3, IgG2a, BD Pharmingen, San Diego, CA), and anti-CD107a-PE (H4A3, IgG1, BD Pharmingen) were also used. Antibodies concentration were adjusted according to the protocol of the manufacturer. Surface phenotype of NK cells was assessed by indirect immunofluorescence using the appropriate mAb followed by PE-conjugated isotype specific goat anti-mouse second reagent (Southern Biotechnology, Birmingham, AL). For perforin detection intracytoplasmic staining of NK cells was performed using cytofix/cytoperm (BD
PharMingen) and labelling with either anti-perforin-PE (δG9, IgG2b, Ancell, Bayport, MN) or a PE-conjugated isotype matched control as previously described. Flow cytometric analysis was performed by a FACSCalibur cytometer (BD Pharmingen).

**Isolation and culture of NK cell populations**

NK cells from healthy donors and FHL patients were purified using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada). Briefly, 5-10x10^6 PBMC mixed with autologous RBC (RBC:PBMC ratio of 30:1) were resuspended in 1mL of 10%FCS-RPMI and were incubated with 50 µL of RosetteSep cocktail for 20 minutes at room temperature (RT). The sample, diluted 2x with medium, was layered on Ficoll-Hypaque gradients and centrifuged. Highly purified NK cells were recovered at the interface with optimal efficiency. NK cells were cultured on irradiated feeder cells in the presence of 2 µg/mL phytohemagglutinin (Sigma-Aldrich, Irvine, United Kingdom) and 100U/mL rIL-2 (Proleukin, Chiron Corp., Emeryville, USA) to obtain proliferation and great expansions of polyclonal NK cell populations.

**Cytolytic assay**

Polyclonal NK cell populations were tested in a 4-h ^51Cr-release assay for cytolytic activity against the erythroleukemia K562, the HLA-class I melanoma FO-1, the HLA-class I B-EBV cell line 721.221 (thereafter termed 221) and the HLA-class I^+ B-EBV cell line AMALA. We also used immature DC (iDC), derived from healthy individuals as previously described, as target cells. Masking of HLA-class I molecules was accomplished with the addition of saturating amounts of A6-136 mAb. For redirected killing assays, the murine mastocytoma FcγRc^+ P815 cell line was used as a target cell in the presence of mAb specific to triggering receptors of IgG isotype at a concentration of 0.5 µg/mL. The E/T ratios are indicated in the text.

**CD107a assay**

We performed the degranulation assay quantifying cell surface CD107a expression, as previously described with minor modifications. Briefly, 2x10^5 polyclonal NK cell populations or 24 hour-IL-2 activated PBMCs cells were co-cultured with 2x10^5 target cells (K562, FO-1, 221 or P815 cells) in 96 V bottom well plates. In each well, containing 200 µl E/T cell suspension, 5 µl of PE conjugated anti-CD107a mAb (BD Pharmingen) were added prior to incubation. Cells were mixed by gentle pipetting.
and incubated for 2 hours at 37°C in 5% CO₂. To induce r-ADCC against P815, NK cells were incubated with 50µl of IgG1 mAbs as indicated in the text. Thereafter, the cells were collected, washed in PBS, and stained with anti-CD3-FITC and anti-CD56-PC5 mAbs for flow cytometric analysis (FACSCalibur, Becton Dickinson). Surface expression of CD107a was assessed in the CD56⁺ cell fraction of either CD3⁻ PBMC or polyclonal NK cell populations.

**NK-cell stimulation and cytokine analysis**

Polyclonal NK cell populations from patients and healthy donors were stimulated as follows. NK cells (1.5x10⁵/well) were cultured overnight in 96 well flat bottom plastic plates (200 µl/well) precoated or not with the anti-NKp30 mAb (F252, 10 µg/mL), the anti-NKp46 mAb (KL247, 10 µg/mL), the anti-NKp44 (KS38, 10 µg/mL), anti-CD16 (c127, 1 µg/mL) or anti-CD56 as control mAb (A6/220, 10 µg/mL). NK cells (1.5x10⁵/well) were also stimulated by overnight co-culture with 221 B-EBV cell line (5x10⁴/well) in U bottom plastic plates (200 µl/well). The culture supernatants were then collected and analyzed for the presence of TNF-α and IFN-γ. Cytokine analysis was carried out using enzyme-linked immunosorbent assay (ELISA) kits from BioSource International (Camarillo, CA) according to the manufacturer’s instructions.

**Immunoblotting**

Polyclonal activated NK cells were washed in PBS and lysed at 2x10⁷ cells/mL in 50mM Tris [tris(hydroxymethyl)aminomethane] HCl [pH8], 150mM NaCl, 1mM MgCl₂, 1% Triton X-100 with complete protease inhibitor (Roche Diagnostics Ltd., United Kingdom) for 15 minutes on ice, vortexing every 5 minutes. Nuclei and membranes were spun out at 13,000rpm for 15 minutes at 4°C. Lysates were resolved by SDS gel electrophoresis (SDS-PAGE) on NuPAGE 4-12% Bis-Tris gels (Invitrogen, Paisley, United Kingdom) under reducing conditions. Proteins were transferred to nitrocellulose membranes (Invitrogen) using a XCellIII blot module (Invitrogen) in 25mM Tris (pH8.3), 192mM glycine and 20% methanol. Membranes were blocked in PBS, 5% milk powder and 0.1% Tween20 for 1 hour at room temperature, incubated for overnight at 4°C with rabbit anti-Munc13-4 antibody raised against aminoacids 1-262 (a gift from Hisanori Horiuchi). Membranes were washed 3 times in PBS/0.1% Tween20 for 10 minutes each and incubated for one hour with HRP-labeled anti-rabbit Ig secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in blocking buffer. Excess HRP was removed
by washing 3 times in PBS/0.1% Tween20 for 10 minutes each and developed for 5
minutes in Supersignal (Perbio Science UK Ltd., CramLington, United Kingdom),
exposed for 1 minute to 1 hour using Biomax Film (Kodak, Sigma-Aldrich). SeeBlue
Plus2 (Invitrogen) was loaded on each gel as molecular weight standards. The
membranes were normalized utilizing a rabbit anti-actin antibody (Sigma-Aldrich).

**Immunofluorescence Microscopy**

NK cells were conjugated with 221 B-EBV target cells and attached to slides in
serum-free RPMI-1640 at 37°C for 15 minutes and stained as previously described.\(^{47}\)
Primary mouse monoclonal antibodies anti-perforin (δG9, BD Biosciences, Oxford,
UK) and anti-tubulin (TAT-1, a gift from Keith Gull, Sir William Dunn School of
Pathology, Oxford University, UK) or rabbit antiserum against Cathepsin D (Upstate,
Lake Placid, NY) and either FITC- or Cy3-labelled secondary antibodies (Jackson
ImmunoResearch, Soham, UK), were utilized. Samples were analysed using a Zeiss
Axioplan 2 microscope (Carl Zeiss Ltd., Hertfordshire, United Kingdom) mounted
with a CoolSnap HQ Camera. Images were processed using Metamorph software
(Molecular Devices, Downington, PA) and AutoDeblur + AutoVisualize software
(AutoQuant Imaging Inc., Waterwliet, NY).
Results

**Perforin and Munc13-4 expression in FHL NK cells.**

Five patients, identified by FACS analysis as perforin-deficient and confirmed by genetic analysis to carry non-sense perforin mutations (FHL2 subtype), were included in this study (Table I). Figure 1 shows the FACS profiles of perforin from a representative FHL2 patient (UPN314, panel C) in comparison to a healthy control (panel A) and a FHL3 patient (UPN336, panel B).

Genetic analysis of Munc13-4 identified eight FHL3 patients with mutations illustrated in Table II. Figure 2 shows the analysis of Munc13-4 expression in activated polyclonal NK cell populations derived from two healthy donors (H1 and H2), four FHL3 patients (UPN237, 336, 293 and 180) and a representative FHL2 patient (UPN314). While equal levels of Munc13-4 are expressed in healthy donors and FHL2 patient, protein expression is not detected in UPN336 and 180, reduced protein levels are detectable in UPN293, in which the predicted protein possesses a 4 amino acid deletion, and only a trace amount of protein is detected in UPN237. These results show that the mutations summarized in Table II result in greatly reduced or complete loss of Munc13-4 protein expression in NK cells.

**Confocal microscopy analysis of cytotoxic granules in perforin and Munc13-4 defective NK cells.**

In order to analyze the perforin localization as well as the polarization of lytic granules in patient or donor NK cells, granules were labeled using antibodies against perforin, cathepsin D and microtubules using an antibody against tubulin. NK cells were visualized either alone or conjugated to the 221 B-EBV cell line. Perforin and cathepsin D co-localise in the same granules in control (Figure 1D) and FHL3 patient UPN336 (Figure 1E), while only cathepsin D is detectable in the FHL2 patient UPN314 (Figure 1F), thus confirming the loss of perforin expression revealed by FACS analysis (Figure 1C). Lytic granules are distributed along microtubules in healthy donor, FHL2 and FHL3 patients (Figure 1G-H) and polarize tightly at the immunological synapse (Figure 1I-N), consistent with previous results in CTL.9
Cytotoxic activity of NK lymphocytes in FHL patients.

Cytolytic activity of freshly derived PBL against K562 target cells (usually referred to as NK activity) was preliminarily tested in FHL3 patients and found to be markedly reduced (A. Santoro et al., submitted). To further characterize this functional impairment, purified NK cells were expanded in IL-2. These activated polyclonal NK cells were tested against a variety of target cells by using a standard ⁵¹Cr-release assay (Figure 3). These included the melanoma FO-1 (panel A, left quadrant) and K562 (not shown) tumor cell lines, the 221 (HLA-class I, panel A right quadrant) and AMALA (HLA-class I⁺, not shown) B-EBV cell lines, and immature dendritic cells (iDC, panel B). In addition the FcγRc⁺ P815 was tested to assess the function of individual activating receptors in redirected killing assay (panel C). NK cells from FHL2 patients and healthy donors were tested for comparison. NK cells from FHL3 patients displayed intermediate levels of cytotoxicity with respect to the high efficiency of killing by NK cells from healthy donors and the inability of killing by perforin-deficient NK cells from FHL2 patients. The defect of FHL3 NK cells was more evident against the B-EBV cell lines 221 and AMALA (upon mAb-mediated masking of HLA class I) than using the tumor cell lines as target cells. Indeed, killing of K562 was clearly impaired only in patient UPN336. FO-1 target cells elicited a better discrimination of killing capability and also in this assay UPN336 appeared the most defective patient. Notably, Munc13-4 expression was completely absent in this patient (Table II).

Immature DC, characterized by low surface expression of HLA-class I molecules, are usually highly susceptible to lysis by normal NK cells, both in allogeneic and autologous combination. Indeed, as shown in Figure 3B, NK cells from healthy donors efficiently killed allogeneic iDC and lysis was not increased by the addition of anti-HLA-class I mAb. Notably, the same iDC were poorly lysed by the NK cells derived from FHL3 patients and restoration of lysis was observed upon mAb-mediated masking of HLA-class I molecules. Although the values did not reach those of normal NK cells, these data indicate that due to an inefficient mechanism of lysis induction, the inhibitory receptors can predominate and elicit a strong inhibition of target cell lysis. As expected, the FHL2 NK cells did not kill even following addition of anti-HLA-class I mAb.⁴⁹
We then assessed the ability of different triggering receptors including NCR, NKG2D, CD16, CD2 and 2B4 to induce killing of P815. In Figure 3C, in UPN293 strong NK cell cytotoxicity could be induced by mAb to NCR (the representative NKp30 is shown), CD16 and NKG2D, although to a lesser extent than NK cells from the healthy control. Remarkably, triggering via CD2 and 2B4, that in normal NK deliver a weaker activation signal, were virtually inactive in UPN293 NK cells. On the other hand, in the perforin deficient NK cells, none of the activation pathways initiated by different receptors resulted in efficient target cell lysis. Taken together, these data support the notion that activated NK cells from FHL3 patients display an impaired cytolytic activity although less marked than in FHL2 patients.

**CD107a expression on FHL patients after co-culture with target cells.**

FHL3 patients are characterized by critical mutations in the gene encoding Munc13-4, a protein essential for cytolytic granule fusion to the cell surface membrane. Therefore, we further analyzed whether the cell surface expression of CD107a molecule, which marks degranulation in NK cells, was altered. Consistent with previous data, resting normal NK cells (CD3− CD56+ gated in PBMC) were stained with anti-CD107a mAb (range of 5-25% CD107a+ cells) when co-cultured with K562 (not shown). The percentage of CD107a+ cells increased when they were incubated overnight with IL-2 before addition of target cells (range 27-75% CD107a+ cells). In order to allow an optimal discrimination between normal and pathological samples, we standardized the assay using a short term IL-2 cell culture. As shown in Figure 4A, NK cells from a representative healthy individual were compared to those from FHL2 (UPN314) and two different FHL3 (UPN293 and 336) patients. While normal NK cells displayed 73% CD107a+ cells, NK cells with a Munc 13-4 defect had only 9% CD107a+ cells with a dim staining. In contrast, perforin deficient NK cells, although unable to lyse K562 target cells, showed a very high proportion (41%) of CD107a+ cells. In Figure 4 (panels B-D), CD107a expression in NK cells from the same donors was also tested using purified polyclonal NK cell populations. These were incubated with different target cells, such as FO-1 (panel B), K562 (not shown), 221 (panel C) and P815 (redirected killing assay using anti-NKp30 mAb, panel D). The highly susceptible FO-1 and K562 tumor cell lines induced a strong CD107a expression in both healthy and perforin deficient NK cells (consistently over 40%).
while in Munc 13-4 deficient NK cells CD107a expression was lower both in terms of percentage of positive cells (18%) and of mean fluorescence intensity (MFI 7 compared to 75 of the healthy donor and 52 of FHL2 patient). Although the B-EBV cell line 221 was less efficient in inducing CD107a expression by NK cells, differences between healthy and perforin deficient NK cells as compared to Munc 13-4 deficient NK cells were still clearly detectable (11% and 22%, respectively, versus 3%). Finally when NK cells from normal individuals were tested against P815 upon addition of mAb specific for various activating receptors, similar data (~ 50% CD107a⁺ cells) were obtained with NKp30, NKp46, NKp44 and CD16 (results of NKp30 triggering are shown in Figure 4D); in controls, in which no mAb was added, CD107a⁺ cells were less than 4%. Thus, in normal NK cells the cytofluorometric analysis using CD107a correlates well with the results of the ⁵¹Cr-release assay (see also Figure 3C). Also in this case, perforin deficient NK cells displayed normal levels of CD107a expression while FHL3 NK cells were clearly defective. Fig. 4E further documents that the defect of CD107a expression (upon exposure to various target cells) is a common feature to all FHL3 patients, analyzed as a group as well as individually. In particular CD107a expression was observed in 11.8 ± 7.5% of cells in six FHL3 patients, while it was 48.5 ± 11.5% in nine healthy individuals (p<0.001). The MFI was also significantly lower in the FHL3 patients compared to the controls (9.3 ± 3.1 vs. 42.6 ± 21.9; p<0.001). In contrast to FHL3, the mean values of CD107a expression obtained from three FHL2 patients were even higher than healthy controls both in terms of percentage (63.3%) and MFI (86.6).

Altogether, these data obtained by flow-cytometry show that the pattern of CD107a expression represents a novel tool to identify, among patients with FHL, the defect of degranulation which is characteristic of Munc 13-4 deficiency. Importantly, in FHL2 NK cells, whose granules lack perforin, the degranulation pattern is normal.

**Cytokine production from NK cells in FHL patients.**

Polyclonal NK cell populations derived from four FHL3 and four FHL2 patients were examined for cytokine production and compared to healthy donors. TNF-α (Figure 5) and IFN-γ (not shown) production was induced by NK cell triggering via anti-NKp30 mAb (panel A) or by overnight co-culture with 221 B-EBV cell line (panel B). NKp30 stimulation induced the production of high amounts of both cytokines with no
substantial differences between patients and healthy donors. Similar data were obtained upon cell stimulation via NKp46, NKp44 and CD16 (not shown). The basal cytokine production (i.e. NK cells incubated with no mAb or with anti-CD56 mAb) was similarly low in patients and healthy individuals. On the other hand, when NK cells were cultured with 221 cells, a clear difference existed between patients, characterized by high production, and healthy controls, characterized by low production. This difference could be explained by the fact that, in contrast to normal NK cells, those from FHL patients are inefficient in killing the B-EBV cells (see Figure 3A), which therefore remain alive and continue to provide a stimulatory signal to NK cells. Normal NK cells, efficiently killing 221, rapidly eliminate the source of stimulating interactions. That this might be a likely explanation is further supported by the finding that target cells which are partially lysed by FHL3, such as FO-1 and P815 in the presence of anti-NKp30 mAb (not shown), induced in FHL3 NK cells cytokine production only slightly above normal cells. In addition NK cells from FHL2 patients that do not lyse any of the target cells analyzed, consistently produced high amounts of TNF-α when co-cultured with these targets.
Discussion
The present study focuses on the NK cell function in patients with different FHL subgroups. We show that, by using appropriate target cells, it is possible to detect both perforin and Munc13-4 defects. Remarkably, we first report that the analysis of CD107a, a marker of granule exocytosis (expressed at the surface of NK cells after their interaction with suitable target cells), in combination with the detection of intracytoplasmic perforin, allows a rapid discrimination between FHL2 and FHL3.

For most patients with FHL, viral infection may represent a serious challenge sometimes difficult to overcome. During viral infection, the balance between the virus and the host may vary and result in different scenarios. When the cytotoxic response is rapid and efficient, infected cells are rapidly killed and the infection terminated as a result of the clearance of the virus. If the killing mechanism is inefficient, as in the case of FHL patients, both T and NK cells become activated and undergo proliferation, however, they fail to kill infected cells and don’t arrest virus dissemination. On the other hand, since the source of antigen stimulation is not removed, a persistent T and NK cell activation occurs and results in the production of large amounts of cytokines, including IFN-\(\gamma\), granulocyte-macrophage colony-stimulating factor (GM-CSF) (i.e. major macrophage activators) and TNF\(\alpha\). In turn, the macrophage homing to the sites of T and NK cell triggering and activation results in tissue infiltration and in the production of high levels of primary inflammatory cytokines, including TNF\(\alpha\), IL-1, and IL-6, which play a major role in tissue damage and in the various clinical symptoms. It is of note that HLH represents a clinical syndrome, resulting from inefficient cytolytic function and macrophage hyperactivation, which may be common not only to FHL subtypes but also to different congenital immune defects (including Chediak-Higashi and Griscelli syndrome). On the other hand, a different pathogenic mechanism occurs in male children with X-linked lymphoproliferative disease (XLP), a condition which, in some situations, may be barely distinguishable from HLH.\(^{20,51}\) Thus, XLP patients are unable to control Epstein-Barr virus (EBV) infection as a consequence of a major dysfunction of 2B4 receptor which exerts inhibitory instead of activating function.\(^{52}\) Remarkably, our present data provide a simple in vitro model which mimics the effect of the inability of effector cells to clear the source of (antigen) stimulation. Thus, NK cells from healthy individuals, when co-cultured with the B-EBV cell line 221,
released small amounts of cytokines, while those isolated from FHL2 and FHL3 patients were consistently high producers. The explanation for this difference is that normal NK cells, but not FHL NK cells, rapidly killed target cells thus removing the source of NK cell stimulation. Notably, 221 target cells were largely viable after 24 hours of co-culture with FHL NK cells. Remarkably, in FHL patients the overwhelming activation of the immune system may also result from the marked impairment of NK-mediated killing of iDC (see Fig. 3B and ref. 49). Indeed, the lack of an efficient DC editing may further lead to an excessive T cell activation and cytokine release. It should be stressed that potential target cells for NK-mediated lysis *in vivo* are likely to be mostly represented by virus-infected cells and iDC. Therefore, the insight into NK cell function provided by our present study may be more representative of the pathophysiology of HLH.

Activated NK cells derived from various FHL3 patients showed a variable degree of cytotoxic activity against highly susceptible tumor targets. This correlated with the amounts of Munc13-4 protein detected by western blot analysis. Our results show that even trace amounts of Munc13-4 protein, associated with certain mutations, were sufficient to allow some killing, while the complete absence (e.g. UPN336) led to a marked cytolytic defect. FHL2 NK cells were unable to lyse any target, included the highly susceptible ones.

In addition to providing information useful for the pathophysiology of FHL and for a functional correlation with the different mutations, our data may impact on the diagnosis of this disease. Indeed, we report the first use of surface CD107a expression as a novel screening tool for identification, among FHL patients, those with Munc13-4 defect (FHL3). It is conceivable that other diseases characterized by defects in granule exocytosis may display the same CD107a defective pattern. However, these diseases (e.g. Griscelli syndrome or Chediak-Higashi) can be distinguished from FHL on a clinical ground. Upon co-culture with target cells, NK lymphocytes from FHL3 patients showed a sharply lower frequency and MFI of CD107a staining compared to healthy controls. Thus, the defect of granule exocytosis could be clearly detected. Differently, FHL2 NK cells, lacking perforin in their granules, showed a normal pattern of CD107a staining. Although the use of purified activated NK cell populations may increase the discrimination power of this test, it is remarkable that even PBMC (24 hours IL-2 activated) could be used to reveal a defect of granule exocytosis. Thus, the combined use of surface expression of CD107a, together with
intracytoplasmic staining with anti-perforin mAb44 allows to promptly dissect FHL3 and FHL2 defects by the simple analysis of PBMC, so directing further genetic analysis.
Table I. Perforin mutations in FHL2 patients

<table>
<thead>
<tr>
<th>UPN</th>
<th>Mutations</th>
<th>Predicted effect</th>
<th>Perforin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>** del 50T</td>
<td>L17 FsX</td>
<td>Absent</td>
</tr>
<tr>
<td>235</td>
<td>* del 847-852</td>
<td>del L283-L284 A91V</td>
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<tr>
<td></td>
<td>* C272T</td>
<td>del L283-L284 A91V</td>
<td></td>
</tr>
<tr>
<td>256</td>
<td>* C272T G695A</td>
<td>A91V R232H</td>
<td>Absent</td>
</tr>
<tr>
<td>314</td>
<td>** G1122A</td>
<td>W374X</td>
<td>Absent</td>
</tr>
<tr>
<td>306</td>
<td>* G1122A C657A</td>
<td>W374X Y219X</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Perforin gene mutations in both alleles are listed in the table including five FHL patients.
UPN indicates unique patient number; double asterisk (**), homozygous mutation;
single asterisk (*), heterozygous mutation; X, stop; Fs, frameshift.
Perforin expression was tested by flow-cytometry.
Table II. Munc 13-4 mutations in FHL3 patients

<table>
<thead>
<tr>
<th>UPN</th>
<th>Mutations</th>
<th>Predicted effect</th>
<th>Munc 13-4 expression</th>
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<tr>
<td>180</td>
<td>*</td>
<td>G175A, 753+1G &gt;T</td>
<td>A59T splice error</td>
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<tr>
<td></td>
<td>**</td>
<td></td>
<td></td>
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<tr>
<td>277</td>
<td>*</td>
<td>del 3082C, G1241T, C2782T</td>
<td>1028 Fs R414L, R928C</td>
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<tr>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>249</td>
<td>**</td>
<td>A1847G</td>
<td>E616G</td>
</tr>
<tr>
<td>237</td>
<td>*</td>
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<td>E616G R414L, R928C</td>
</tr>
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<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>*</td>
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<td>E616G splice error</td>
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<td></td>
<td>*</td>
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<tr>
<td>289</td>
<td>*</td>
<td>A610G, C2650T ins G3226</td>
<td>M204V, Q884X 1076 FsX</td>
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<td></td>
<td>*</td>
<td></td>
<td></td>
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<tr>
<td>293</td>
<td>**</td>
<td>del 1822-1833 del V608-A611</td>
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<tr>
<td>336</td>
<td>**</td>
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<td>A59T 147 Fs</td>
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</table>

Munc13-4 gene mutations in both alleles are listed in the table including eight FHL patients.

UPN indicates unique patient number; double asterisk (**), homozygous mutation; single asterisk (*), heterozygous mutation; X, stop; Fs, frameshift. Munc13-4 expression was tested by western blot.
Table III. Main characteristics of FHL patients

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<th>UPN</th>
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<th>235</th>
<th>256</th>
<th>314</th>
<th>306</th>
<th>180</th>
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<td>2 m</td>
<td>4 m</td>
<td>2 m</td>
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</tbody>
</table>

UPN indicates unique patient number; HSCT, allogeneic hematopoietic stem cell transplantation; DoD, dead of disease; DoT, dead of toxicity; and ND, not determined.
Figure 1: Perforin expression and granule polarization FHL patients compared to control.

Polyclonal activated NK cells derived from healthy donor (A,D,G,L), FHL3 UPN336 (B,E,H,M) and FHL2 UPN314 (C,F,I,N) were analysed by flow cytometry (A-C) and confocal microscopy (D-N). Filled curves indicate perforin expression, while open curves indicate isotypic control (A-C). Immunofluorescent staining of perforin (green) and cathepsin D (red) (D-F) or cathepsin D (red) and tubulin (green) (G-N) is analysed in isolated NK cells (D-I) or conjugates between NK and target 221 (L-N). Bar indicates 10 µm.
Figure 2: Munc 13-4 expression in control and FHL NK cells.
Cell extracts from polyclonal NK cells of healthy donors (H1, H2), FHL3 patients (UPN237, 336, 293, 180) and FHL2 patient (UPN314) were analyzed by western blot with anti-Munc 13-4 antibody. Blots were re-probed with anti-actin antibody. Molecular weight standards are shown on the left (kD).
Figure 3: Cytotoxic activity in FHL patients.

Using the standard $^{51}$Cr-release assay, polyclonal activated NK cells derived from FHL3 and FHL2 patients were compared to those from age-matched healthy individuals for cytolytic activity against various target cells. (A) NK cells from a large group of healthy donors (mean of values, closed squares, with standard deviations), from five different FHL3 (open symbols and cross) and two different FHL2 patients (closed circles and triangles) were tested against the melanoma FO-1 and the B-EBV cell line 221 at different E:T ratio, as indicated. (B) NK cells from two representative healthy, FHL3 and FHL2 individuals were tested against iDC derived from a single allogeneic healthy individual either in the absence (white bar) or in the presence (black bar) of anti-HLA-class I mAb. The E:T ratio used was 10:1. (C) NK cells from one representative healthy, FHL3 (UPN293) and FHL2 (UPN314) individuals were tested against the Fcγ-Re$^+$ P815 in the absence or in the presence of mAb to different triggering receptors, as indicated. The E:T ratio used was 4:1.
Figure 4: CD107a surface expression on NK cells, upon target interaction, identifies the Munc 13-4 defect.

(A) PBMC from a representative healthy donor, a FHL2 patient (UPN314) and two FHL3 patient (UPN293 and 336) were overnight cultured in the presence of IL-2 and then co-cultured with K562. (B-D) Polyclonal activated NK cell populations derived from the same donors were co-cultured with FO-1 (B), 221 (C) and P815 with anti-NKp30 mAb (D). Cells were stained with anti-CD56-PC5 mAb and anti-CD107a-PE mAb and then analyzed by double fluorescence gating on CD56+ cells. Numbers indicate the percentage of CD107a+ cells. In panel E, histograms refer to the percentage of CD107a+ cells (gray bar) and to the mean fluorescence intensity (MFI) of CD107a surface expression (black bar) considering CD56+ cells in a group of FHL3 patients (mean of six ± SD) compared to healthy individuals (mean of nine ± SD) and FHL2 patients (mean of three) after co-culture with FO-1. In FHL3 patients, both percentage and MFI were significantly lower than in healthy controls (p< 0.001; Student t test). FHL3 patients are also shown individually.
Figure 5: TNF-α production by FHL patients compared to healthy donors.

Polyclonal NK cell populations from four different healthy donors, FHL3 (UPN249, 237, 225 and 289) and FHL2 (UPN210, 235, 256 and 314) patients were stimulated overnight by plastic-bound anti-NKp30 mAb (A) or by co-culture with 221 cell line (B). Supernatants were harvested and analyzed by specific ELISA for their TNFα content. Bars represent the mean of values within a group. Differences among groups were not significant in panel A (p>0.05), while both FHL3 and FHL2 were different (p<0.05) from healthy group in panel B (Kruskal-Wallis test).
References


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Analysis of Natural killer cell function in familial hemophagocytic lymphohistiocytosis (FHL). Defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease

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