# A CD57$^{+}$ CTL degranulation assay effectively identifies familial hemophagocytic lymphohistiocytosis type 3 patients

Masayuki Hori [1], Takahiro Yasumi [1], Saeko Shimodera [1], Hirofumi Shibata [1], Eitaro Hiejima [1], Hirotsugu Oda [1,2], Kazushi Izawa [1], Tomoki Kawai [1], Masataka Ishimura [3], Naoko Nakano [4], Ryutaro Shirakawa [5], Ryuta Nishikomori [1], Hidetoshi Takada [3], Satoshi Morita [6], Hisanori Horiuchi [5], Osamu Ohara [2,7], Eiichi Ishii [4], and Toshio Heike [1]

[1]Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Laboratory for Integrative Genomics, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan; [3]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [4]Department of Pediatrics, Ehime University Graduate School of Medicine, Toon, Japan; [5]Department of Molecular and Cellular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan; [6]Department of Biomedical Statistics and Bioinformatics, Kyoto University Graduate School of Medicine, Kyoto, Japan; [7]KAZUSA DNA Research Institute, Kisarazu, Japan

Corresponding Author: Takahiro Yasumi

Department of Pediatrics, Kyoto University Graduate School of Medicine

54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

E-mail: yasumi@kuhp.kyoto-u.ac.jp

Tel: +81-75-751-3291 Fax: +81-75-752-2361

**Abstract**

*Purpose*

Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) is a genetic disorder that results in immune dysregulation. It requires prompt and accurate diagnosis. A natural killer (NK) cell degranulation assay is often used to screen for FHL3 patients. However, we recently encountered two cases of late-onset FHL3 carrying novel *UNC13D* missense mutations: in these cases, the degranulation assays using freshly isolated and interleukin (IL)-2-activated NK cells yielded contradictory results. Since the defective degranulation of $CD57^+$ cytotoxic T lymphocytes (CTLs) in these cases was helpful for making the diagnosis, we assessed whether the $CD57^+$ CTL degranulation assay more effectively identified FHL3 patients than the NK cell assays.

*Methods*

Forty additional patients with hemophagocytic lymphohistiocytosis were prospectively screened for FHL3 by measuring the perforin expression in NK cells and the the expression of Munc13-4, syntaxin-11, and Munc18-2 in platelets, and by performing NK cell and CTL degranulation assays. The results were confirmed by genetic analysis.

*Results*

The freshly isolated NK cell degranulation assay detected FHL3 patients with high sensitivity (100%) but low specificity (71%). The IL-2-stimulated NK cell assay had improved specificity, but 3 out of the 31 non-FHL3 patients still showed degranulation below the threshold level. The $CD57^+$ CTL degranulation assay identifying FHL3 patients with high sensitivity and specificity (both 100%).

*Conclusions*

The $CD57^+$ CTL degranulation assay more effectively identified FHL3 patients than the NK cell-based assays.

**Key words:** Familial hemophagocytic lymphohistiocytosis type 3; Lysosomal degranulation defect; Functional screening assay; *UNC13D*

**Introduction**

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening syndrome that is characterized by immune dysregulation and hyper-inflammation. It is also histologically characterized by the presence of benign hemophagocytic macrophages [1-3]. HLH is classified into primary (genetic) or secondary (acquired) forms, but this distinction is difficult to make in clinical practice [1, 2].

Familial hemophagocytic lymphohistiocytosis (FHL) is the main form of primary HLH. Several FHL mutations have been identified, namely, in the genes encoding perforin (*PRF1*; FHL2) [4], Munc13-4 (*UNC13D*; FHL3) [5], syntaxin-11 (*STX11*; FHL4) [6], and Munc18-2 (also known as syntaxin-binding protein 2) (*STXBP2*; FHL5) [7, 8]. Perforin is an effector molecule that is contained in the cytolytic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Munc13-4, syntaxin-11, and Munc18-2 are involved in the intracellular trafficking or the fusion of these granules to the plasma membrane and the delivery of their contents into target cells. Thus, the hallmark finding of FHL is the defective cytotoxic activity of CTLs and NK cells [9]

The early diagnosis of FHL is clinically important because its treatment strategy differs greatly from that of secondary HLH. While aggressive immunosuppressive therapy is required for both the primary and secondary forms of HLH in the initial period, it can be gradually tapered for most secondary HLH cases once the clinical remission is achieved. By contrast, strong maintenance therapy followed by hematopoietic stem cell transplantation (HSCT) is mandatory for patients with FHL [1, 2].

While certain combinations of common laboratory parameters are useful for identifying patients with a high possibility of FHL, but functional and molecular analyses are mandatory for confirming the diagnosis [10, 11]. A reliable way to screen for FHL2 is to use flow cytometry to detect perforin expression in NK cells [12, 13]. Functional screening for FHL3-5 involves the detection of CD107a expression on the surface of NK cells; this measures the release of cytolytic granules [7, 8, 14-16]. However, a considerable proportion of secondary HLH patients also exhibit abnormal NK cell degranulation and some patients

with FHL3-5 show normal NK cell degranulation after stimulation with interleukin (IL)-2 [16, 17].

Another possibility is to measure CTL degranulation. While such a CTL-based assay has been described, the methodology is not standardized and contradictory results have been reported [16-18]. Nevertheless, numerous studies have shown that the CTL expressing CD57 have high cytotoxic potential [19]. It was also recently reported that CTL expression of CD57 correlates strongly with the intracellular expression of cytolytic molecules such as perforin and granzymes [20] and is a measure of the degranulation capacity of CTLs [21]. Thus, it is proposed that analyzing $CD57^+$ CTL degranulation may be useful for detecting patients with defective cytolytic granules release. However, the usefulness of this method has not been evaluated.

In this report, we describe two late-onset FHL3 patients carrying novel *UNC13D* missense mutations. Notably, these patients exhibited normal IL-2-activated NK cell degranulation but had defective $CD57^+$ CTL degranulation. The latter observation was helpful for diagnosing these patients. Consequently, to test the ability of the $CD57^+$ CTL degranulation assay to detect FHL3 patients, we prospectively screened HLH patients with this assay. We found that this assay distinguished the FHL3 patients from patients with other forms of HLH with high sensitivity and specificity.

## Materials and Methods

*Patients*

Two late-onset FHL3 cases were diagnosed in 2012 (Patients 1 and 2). Thereafter, in February 2013–April 2014, prospective FHL screening was performed on 40 additional patients who were suspected by their referring physicians to have FHL (Patients 3–42). As a control, blood obtained from healthy adults at the time of patient sampling was shipped for screening along with the patient samples. None of the patients had any sign of oculo-cutaneous albinism or had giant granules in their peripheral blood leucocytes. FHL was screened by measuring perforin expression in NK cells, by measuring Munc13-4, syntaxin-11,

and Munc18-2 expression in platelets, and by NK cell and CTL degranulation assays. Genetic analysis was performed in all patients to confirm the results. The characteristics of the enrolled patients and the genetic defects detected in the FHL patients are summarized in Tables 1 and 2, respectively. Informed consent was obtained from the patients and their parents in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

*Protein expression assays*

All patients were assessed for perforin expression by their NK cells and their platelet expression of Munc13-4, syntaxin-11, and Munc18-2 as previously described, with some modifications [22, 23]. To determine the effect of the *UNC13D* missense mutations observed in the two late-onset FHL3 patients on Munc13-4 protein expression, FLAG-tagged cDNA carrying wild-type or missense mutated *UNC13D* sequences were constructed and transiently transfected into HEK293T cells. After overnight culture, the cells were harvested with or without additional incubation in the presence of 0.35mM cycloheximide. Cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the fractionated proteins were electro-transferred onto polyvinylidene fluoride membranes. The membranes were blocked overnight in blocking buffer (5% skim milk) and incubated for 1 hour at room temperature with anti-FLAG antibodies, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Specific bands were visualized by the standard enhanced chemiluminescence method.

*Antibodies*

Rabbit polyclonal antibodies specific for human Munc13-4 and syntaxin-11 proteins were described previously [23, 24]. Rabbit polyclonal antibodies against human Munc18-2 protein were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit polyclonal anti-integrin αIIb (Santa Cruz Biotechnology), mouse polyclonal anti-β-actin

(Sigma Aldrich, St. Louis, MO), and mouse monoclonal anti-FLAG (Sigma Aldrich) antibodies served as primary antibodies in Western blotting. The monoclonal antibodies used in the flow cytometric analyses were fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (eBioscience, San Diego, CA), phycoerythrin (PE)-Cy7-conjugated anti-CD3 (Beckman Coulter, Brea, CA), FITC-conjugated anti-CD8 (eBioscience), V500-conjugated anti-CD16 (BD Biosciences), PE-conjugated anti-CD41a (BD Biosciences, San Jose, CA), allophycocyanin (APC)-conjugated anti-CD56 (Beckman Coulter), APC-conjugated anti-CD57 (BD Biosciences, San Jose, CA), PE-conjugated anti-CD107a (eBioscience), and PE-conjugated anti-perforin (eBioscience).

*Mutation analyses*

Genomic DNA was isolated from the peripheral blood mononuclear cells (PBMCs) of the patients by standard procedures. Primers were designed to amplify the coding exons and adjacent intronic sequences of the *PRF1*, *UNC13D*, *STX11*, *STXBP2*, *SH2D1A*, *BIRC4*, and *ITK* genes. Primers that would detect the deep intronic mutation in the UNC13D intron 1 were also designed. Primer sequences are available upon request. The amplified products were sequenced with an Applied Biosystems ABI3130 Genetic Analyzer (Life Technologies, Carlsbad, CA) or with a GS Junior System (Roche, Basel, Switzerland).

*Lysosomal degranulation assays*

To quantify lysosome exocytosis by NK cells, $2\times10^5$ PBMCs that were freshly isolated or stimulated for 36–48 hours with IL-2 (50 U/mL), were cultured with or without $2\times10^5$ K562 cells and incubated in complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum) for 2 hours at 37°C in 5% $CO_2$. For CTL degranulation analyses, $2\times10^5$ PBMCs stimulated for 36–48 hours with IL-2 (50U/mL) were cultured with $2\times10^5$ P815 cells with or without 0.5 μg/mL anti-CD3 mAb (OKT3). The cells were resuspended in phosphate-buffered saline supplemented with 2% fetal calf serum and 2 mM EDTA; stained with anti-CD3, anti-CD8, anti-CD16, anti-CD56, anti-CD57, and

anti-CD107a monoclonal antibodies, and then analyzed by flow cytometry.

*Statistical analyses*

Receiver operating characteristic (ROC) analysis was performed to determine the optimal threshold of each assay that would discriminate FHL3 patients from other HLH patients with the greatest sum of sensitivity and specificity. Laboratory-defined thresholds were also determined (*i.e.*, the lowest degranulation value observed in the control subjects). The sensitivity and specificity of each assay were evaluated on the basis of these thresholds. The controls were excluded from the analyses. In the case of patients who were tested multiple times with particular assays, only the data from the first evaluation were included in the statistical analyses.

**Results**

*Late-onset FHL3 patients*

Patient 1 was a 90-months-old boy who developed HLH after a *Mycoplasma pneumoniae* infection (Table 1). Oral prednisolone was effective in controlling the disease, but subsequent tapering of the drug resulted in disease recurrence that associated with cerebral symptoms. Genetic analysis identified compound heterozygous *UNC13D* mutations, namely, c.754-1G>C on the paternal allele and c.2759A>G (p.Y920C) on the maternal allele (Table 2). Patient 2 was a girl who developed HLH associated with the abnormal infiltration of non-malignant lymphocytes in a vertebra at the age of 190 months (Table 1). She also developed cerebral symptoms but responded well to oral prednisolone therapy. *UNC13D* sequencing revealed that she carried a c.1992+1G>A mutation on the paternal allele and two missense mutations, namely, c.767G>A (p.R256Q) and c.1240C>T (p.R414C), on the maternal allele (Table 2).

Since the missense mutations found in the patients were novel, the diagnosis of FHL3 could not be made with certainty. Moreover, although the freshly isolated PBMCs of both cases exhibited decreased NK cell degranulation, their IL-2-stimulated NK cells released

normal levels of cytolytic granules (Figure 1A and B). Nevertheless, they both exhibited defective degranulation of their $CD57^+$ CTLs (Figure 1C) and reduced expression of platelet Munc13-4 protein. These findings strongly supported the diagnosis of FHL3 (Figure 1D). We therefore assessed whether the *UNC13D* missense mutations in our patients were responsible for their reduced platelet Munc13-4 expression by transfecting HEK293T cells with FLAG-tagged cDNA carrying wild-type or missense *UNC13D* sequences. Indeed, the c.2759A>G mutation in Patient 1, and the c.1240C>T (but not the c.767G>A) mutation in Patient 2, were responsible for the reduced Munc13-4 protein expression (Figure 1E). This confirmed the diagnosis of FHL3 in Patients 1 and 2.

*Comparison of degranulation assays*

Given that the $CD57^+$ CTL degranulation assay more effectively identified our late-onset FHL3 patients than the NK cell-based assays, we compared these three assays prospectively with 40 additional pediatric HLH patients who were recruited after Patient 1 and 2 were diagnosed (Table 1). Six of the forty patients (Patients 3–8) had FHL3. None of these patients carried missense *UNC13D* mutations (Table 2). As shown in Figure 2A, the freshly stimulated NK cells from the six FHL3 patients exhibited decreased cytolytic granule release. However, the NK cells from the non-FHL3 patients also showed some defects in degranulation. When using the laboratory-defined threshold of 10%, the degranulation assay with freshly stimulated NK cells was found to discriminate FHL3 patients from other HLH patients with a sensitivity of 100% and a specificity of 68%. ROC analysis showed that the optimum threshold value was 9.2%. When this value was used as the discriminatory threshold, the specificity improved to 71% without changing the sensitivity.

While IL-2 stimulation restored the degranulation of NK cells from the non-FHL patients in all cases, the IL-2-stimulated NK cells from a FHL3 patient (Patient 5), similar to the IL-2-stimulated NK cells from Patients 1 and 2, showed normal degranulation levels. The laboratory-defined threshold of 30% showed that the IL-2-stimulated NK cell degranulation assay discriminated FHL3 patients from other HLH patients with a specificity of 100% but a

low sensitivity of 63%. ROC analysis showed that the optimum threshold value was 44.7%. When this threshold was used, the sensitivity improved to 100%; however, the specificity decreased to 90% (Figure 2B).

By contrast, when the laboratory-defined threshold of 25% was used for $CD57^+$ CTL degranulation assay, this assay discriminated FHL3 patients from other HLH patients with a sensitivity of 100% and a specificity of 94%. ROC analysis indicated that the optimum threshold was 11.5%. When this value was used, both the sensitivity and specificity were 100% (Figure 2C).

**Discussion**

FHL is a life-threatening inherited immune disorder that is caused by mutations in genes that participate in the cytotoxic activity of lymphocytes. The inability to clear the antigenic stimulus hyper-activates cytolytic lymphocytes. This results in over-production of inflammatory cytokines, which in turn leads to sustained inflammation and activation of macrophages [1, 2, 9]. The ability to rapidly screen for FHL rapidly would facilitate the initiation of life-saving immunosuppressive therapy and the preparations for HSCT.

The lysosomal exocytosis assay is a comprehensive method that is used to identify patients with a degranulation defect. It has been used to screen for FHL3–5 and hereditary HLH syndromes associated with oculo-cutaneous albinism [7, 8, 14-16]. In the present study, the FHL3 patients exhibited reduced degranulation of resting NK cells. However, as was shown by a previous study [16], non-FHL3 patients also exhibited some defects in resting NK cell degranulation (Figure 2A). While IL-2 stimulation restored the degranulation of the NK cells from these non-FHL3 patients, the degranulation of the IL-2-stimulated NK cells from some of the FHL3 patients was comparable to that seen in the control subjects (Figure 2B). By contrast, the decreased degranulation of $CD57^+$ CTLs was more indicative of FHL3 patients (Figure 2C). Specifically, when laboratory-defined thresholds were used to discriminate FHL3 patients from the other HLH patients, the fresh NK cell degranulation assay had a sensitivity and specificity of 100% and 68%, respectively, while the

IL-2-stimulated NK cell degranulation assay had a sensitivity and specificity of 63% and 100%, respectively. Thus, when using laboratory-defined thresholds, the $CD57^+$ CTL assay was as sensitive as the fresh NK cell assay and a bit less specific than the IL-2-stimulated NK cell assay. However, while 18 patients in total exhibited decreased degranulation below the threshold levels of either NK cell assay, only ten patients exhibited decreased degranulation of $CD57^+$ CTLs (Table 1). This is clinically important because it means that the $CD57^+$ CTL assay essentially made genetic testing unnecessary for eight patients. Moreover, when ROC-determined optimal thresholds were used to discriminate FHL3 patients from the other HLH patients, the fresh NK cell degranulation assay had a sensitivity and specificity of 100% and 71%, respectively, while the IL-2-stimulated NK cell degranulation assay had a sensitivity and specificity of 100% and 90%, respectively. By contrast, the sensitivity and specificity of the $CD57^+$ CTL degranulation assay were both 100%. Notably, one patient with other forms of HLH exhibited $CD57^+$ CTL degranulation levels just above the optimum threshold (Patient 21 in Table 1): 1 week later, however, this patient exhibited normal degranulation levels. This was in clear contrast to the re-evaluated FHL3 patients (Patients 1–4 in Table 1), all of whom showed sustained defects in $CD57^+$ CTL degranulation. Although it is possible that immunosuppressive therapies may affect CTL function, the two FHL2 patients who had been treated with multiple immunosuppressive drugs exhibited normal lysosomal degranulation. Thus, these treatments had minimal effects on the assay. Taken together, we propose that the $CD57^+$ CTL degranulation assay effectively identifies FHL3 patients.

A workflow for the diagnosis of primary HLH has been proposed on the basis of a study by Bryceson *et al.* on HLH patients in Europe. The assays using NK cells were the mainstay screening methods for identifying patients with a defect in cytolytic granule exocytosis. The study reported that these assays had a higher specificity than the assays reported in the current study [16]. However, in the study by Bryceson *et al.*, secondary HLH patients were defined as patients who developed a single episode that fulfilled the clinical criteria for HLH and exhibited sustained complete remission for at least 6 months after

completing HLH therapy; patients with a refractory HLH course were excluded from the statistical analysis. Examination of these excluded patients showed that many had reduced NK cell degranulation [16]. Since the patient cohort in the present study included many patients with a severe and refractory course of HLH, we believe that the results of our analysis are similar to those reported by Bryceson *et al*.

The current study suggests that the $CD57^+$ CTL degranulation assay may be more useful for diagnosing FHL3 than the assays employing NK cells. However, several issues must be addressed before it can serve as a standard method. One limitation of this assay is that neonates and young infants have very few number of CD57-expressing CTLs in their peripheral blood [25]. Indeed, we could not perform the analysis in two non-FHL patients due to the extreme paucity of this cell population (Table 1). However, all FHL patients in this study had substantial numbers of $CD57^+$ CTLs in their peripheral blood. This probably reflects the pathophysiology of the disease. Another limitation of the current study was the lack of patients with other forms of genetic degranulation defects; this reflects their extreme rarity. While a patient with *BIRC4* deficiency (X-linked lymphoproliferative syndrome type 2) was included in our cohort (Table 1), this hereditary HLH syndrome does not associate directly with cytolytic defects [26, 27]. Further evaluations are required to determine whether the $CD57^+$ CTL degranulation assay is useful for screening FHL4, FHL5, and hereditary HLH syndromes with oculo-cutaneous albinism. Notably, all FHL3 patients who have been diagnosed at our laboratory (including 8 patients presented in this study) lack or have significantly reduced platelet Munc13-4 protein expression (data not shown). This suggests that the detection of Munc13-4 expression is another useful method for screening for FHL3.

The fact that two late-onset FHL3 cases had relatively well-preserved activated NK cell degranulation yet still had poor $CD57^+$ CTL degranulation suggests that Munc13-4 protein may play different roles in the lysosomal degranulation of the two cell subsets. While the molecular mechanism underlying this phenomenon is unclear, we speculate that the preserved degranulation capacity of NK cells may have influenced the clinical picture of the patients. CTLs and NK cells play distinct roles in the pathogenesis of FHL. Analysis of

perforin-deficient mice showed that CTLs, but not NK cells, are necessary for the development of FHL symptoms [28]. Moreover, a recent report shows that NK cell cytotoxicity plays an immunoregulatory role and protects against FHL pathology [29]. In addition, FHL3 patients with atypical presentations, like our two late-onset cases, are reported to have relatively preserved NK cell degranulation, especially after culture with IL-2 [17]. Indeed, another FHL3 patient in our cohort who showed high levels of activated NK cell degranulation (Patient 5 in Table 1) only developed the symptoms of HLH at 4 months of age: this is relatively late for FHL3. We speculate that the preserved cytolytic capacity of NK cells had contributed to the mild clinical courses of these three patients.

## Conclusions

We propose that the $CD57^+$ CTL degranulation assay effectively discriminates FHL3 patients from those with other forms of HLH. Further studies that assess whether these assays are useful for screening patients with FHL4, FHL5, and other forms of degranulation defect-associated hereditary HLH syndromes are warranted.

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## Authorship contributions

Contribution: T.Y., R.N., and T.H. designed the research; M.I., N.N., H.T., and E.I. treated Patients 1 and 2; M.H., S.S., H.S., E.H., K.I., and T.K. performed the degranulation and protein expression assays; H.O. and O.O. performed the genetic analyses; R.S. and H.H. prepared the anti-Munc13-4 and anti-Syntaxin11 antibodies; M.H., T.Y., K.I., T.K., R.N., S.M., and T.H. analyzed and discussed the results; T.Y. and S.M. performed the statistical analysis; M.H. and T.Y. wrote the paper.

**Conflict-of-interest disclosure**

The authors declare no competing financial interests.

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## Table 1. Characteristics and assay outcomes of the enrolled patients

| Patient no. | Final diagnosis | Age (months) at onset | Gender | Fresh NK cell degranulation (%) | Stimulated NK cell degranulation (%) | CD57$^+$ CTL degranulation (%) |
|---|---|---|---|---|---|---|
| 1 | FHL3 | 90 | M | ***5.1*** (***4.8***) | *38.2* (*40.3*) | ***4.7*** (***2.5***) |
| 2 | FHL3 | 190 | F | ***6.9*** (***5.6***) | *43.0* (*38.6*) | ***8.6*** (***9.3***) |
| 3 | FHL3 | 1 | M | ***8.1*** (***8.5***) | ***9.8*** (***21.2***) | ***0.5*** (***2.1***) |
| 4 | FHL3 | 0 | M | ***2.3*** (***5.2***) | ***14.1*** (*33.2*) | ***4.2*** (***3.7***) |
| 5 | FHL3 | 4 | F | ***8.3*** | *43.0* | ***4.5*** |
| 6 | FHL3 | 9 | M | ***3.7*** | ***20.1*** | ***0.5*** |
| 7 | FHL3 | 1 | M | ***8.8*** | ***22.2*** | ***9.7*** |
| 8 | FHL3 | 1 | M | ***8.8*** | ***23.2*** | ***8.0*** |
| 9 | FHL2 | 1 | F | 18.0 | 62.7 | 38.2 |
| 10 | FHL2 | 0 | M | 29.4 | 91.8 | 45.3 |
| 11 | XLP2 | 64 | M | 10.4 | 73.8 | 77.4 |
| 12 | sJIA-MAS | 80 | M | ***5.3*** (18.2) | 46.4 | 28.7 |
| 13 | CAEBV | 156 | M | ***8.5*** | 51.8 | 28.1 |
| 14 | EBV-HLH | 15 | M | 19.4 | 67.9 | 30.2 |
| 15 | EBV-HLH | 17 | M | **9.6** (**9.3**) | 66.2 (70.7) | 28.1 (57.6) |
| 16 | Unknown | 61 | M | 20.2 | 73.3 | 56.0 |
| 17 | EBV-HLH | 14 | M | 11.3 | 46.6 | 74.1 |
| 18 | Unknown | 138 | F | 20.7 | 86.7 | 69.5 |
| 19 | EBV-HLH | 23 | F | AU | AU | 41.7 |
| 20 | Sepsis | 0 | F | ***6.8*** | 64.7 | AU |
| 21 | EBV-HLH | 62 | F | ***4.4*** (32.8) | *31.8* | **13.1** (52.5) |
| 22 | Unknown | 183 | F | 13.3 | 48.8 | 70.6 |
| 23 | sJIA MAS | 9 | F | 24.7 | 73.7 | 41.8 |
| 24 | Unknown | 20 | M | ***7.5*** | *39.6* | **22.5** |
| 25 | Unknown | 11 | M | 29.1 | 79.9 | 57.8 |
| 26 | Unknown | 8 | F | 13.3 | 55.4 | 67.0 |
| 27 | Unknown | 12 | F | 16.2 | 71.0 | 77.2 |
| 28 | EBV-HLH | 18 | F | AU | AU | 98.3 |
| 29 | Unknown | 19 | F | 16.7 | 74.9 | 33.6 |
| 30 | EBV-HLH | 114 | F | 39.1 | AU | 76.4 |
| 31 | HSV-HLH | 0 | F | ***6.9*** | 57.9 | 71.5 |
| 32 | Unknown | 13 | F | AU | 93.8 | AU |
| 33 | sJIA-MAS | 19 | M | 31.5 | 78.7 | 59.7 |
| 34 | Unknown | 39 | F | 24.0 | 81.8 | 83.2 |
| 35 | EBV-HLH | 21 | M | 23.1 | 80.0 | 49.6 |
| 36 | EBV-HLH | 311 | F | ***6.6*** | 53.6 | 40.1 |
| 37 | Unknown | 4 | M | ***4.2*** | 66.1 | 67.8 |
| 38 | Unknown | 14 | F | 22.7 | 86.5 | 56.5 |
| 39 | Unknown | 5 | F | 14.5 | 83.5 | 65.2 |
| 40 | Unknown | 100 | F | 23.3 | 68.6 | 59.3 |
| 41 | CMV-HLH | 0 | M | ***7.9*** | 56.1 | 51.6 |
| 42 | EBV-HLH | 79 | F | 19.4 | *33.0* | 45.5 |

The assay results that were below the laboratory-defined thresholds (10% for the fresh NK, 30% for the IL-2 stimulated NK, and 25% for the $CD57^+$ CTL assays) are shown in bold letters, while those below the ROC-determined optimum thresholds (9.2% for the fresh NK, 44.7% for the IL-2 stimulated NK, and 11.5% for the $CD57^+$ CTL assays) are shown in italics. The numbers in the parentheses indicate the result of the second evaluation and were excluded from the statistical analyses. AU, analysis unavailable; HLH, hemophagocytic lymphohistiocytosis; FHL, familial HLH; XLP, X-linked lymphoproliferative syndrome; EBV, Epstein-Barr virus; CAEBV; chronic active EBV infection; MAS, macrophage activation syndrome; sJIA, systemic-onset juvenile idiopathic arthritis.

**Table 2. Mutations identified in the FHL patients**

| Patient no. | Diagnosis | Gene mutated | 1st allele | 2nd allele |
|---|---|---|---|---|
| 1 | FHL3 | *UNC13D* | c.754-1G>C (S) | c.2759A>G (M) |
| 2 | FHL3 | *UNC13D* | c.1992+1G>A (S) | c.767G>A (M), c.1240C>T (M) |
| 3 | FHL3 | *UNC13D* | c.754-1G>C (S) | c.118-308C>T (T) |
| 4 | FHL3 | *UNC13D* | c.118-308C>T (T) | c.118-308C>T (T) |
| 5 | FHL3 | *UNC13D* | c.2381delT (F) | c.322-1G>A (S) |
| 6 | FHL3 | *UNC13D* | c.754-1G>C (S) | c.1596+1G>C (S) |
| 7 | FHL3 | *UNC13D* | c.118-308C>T (T) | c.1596+1G>C (S) |
| 8 | FHL3 | *UNC13D* | c.118-308C>T (T) | c.1596+1G>C (S) |
| 9 | FHL2 | *PRF1* | c.1090_1091delCT (F) | c.1288_1289insG (F) |
| 10 | FHL2 | *PRF1* | c.1090_1091delCT (F) | c.1A>G (LS) |

Predicted mutation effects are shown in parentheses. S, splice error; M, missense; T, transcriptional dysregulation; F, frameshift; LS, loss of start codon.

**Figure legends**

**Figure 1. FHL screening results of late-onset FHL3 patients**

Freshly isolated (A) or IL-2 stimulated (B) PBMCs were co-cultured with (+) or without (-) K562 cells, and the expression of CD107a on the $CD3^{-}CD16^{+}CD56^{+}$ cell population was evaluated. (C) IL-2-stimulated PBMCs were co-cultured with P815 cells in the presence (+) or absence (-) of an anti-CD3 antibody and the expression of CD107a on the $CD3^{+}CD8^{+}$ cell population was evaluated. (D) Munc13-4 protein expression in the platelets of the patients was evaluated by Western blotting. (E) HEK293T cells were transfected with FLAG-tagged cDNA carrying wild-type or missense mutated *UNC13D* sequences. After overnight culture, the cells were harvested with or without additional incubation in the presence of cycloheximide for 0, 5, and 10 hours. The Munc13-4 protein expression levels were then analyzed by Western blotting. WT, wild type. Representative results of two independent experiments are shown.

**Figure 2. Sensitivity and specificity with which the NK and $CD57^{+}$ CTL degranulation assays discriminate FHL3 patients from other HLH patients**

The figures show the results of lysosomal degranulation assays and their ROC curves using the freshly isolated NK cells (A), IL-2 stimulated NK cells (B), and of IL-2 stimulated $CD57^{+}$ CTLs (C) of the enrolled patients. Solid lines indicate the laboratory-defined threshold values while dashed lines indicate the optimum threshold values that were determined by ROC analyses to discriminate FHL3 patients from other HLH patients. Closed circles indicate Patients 1 and 2. nFHL, non-FHL.

Figure 1

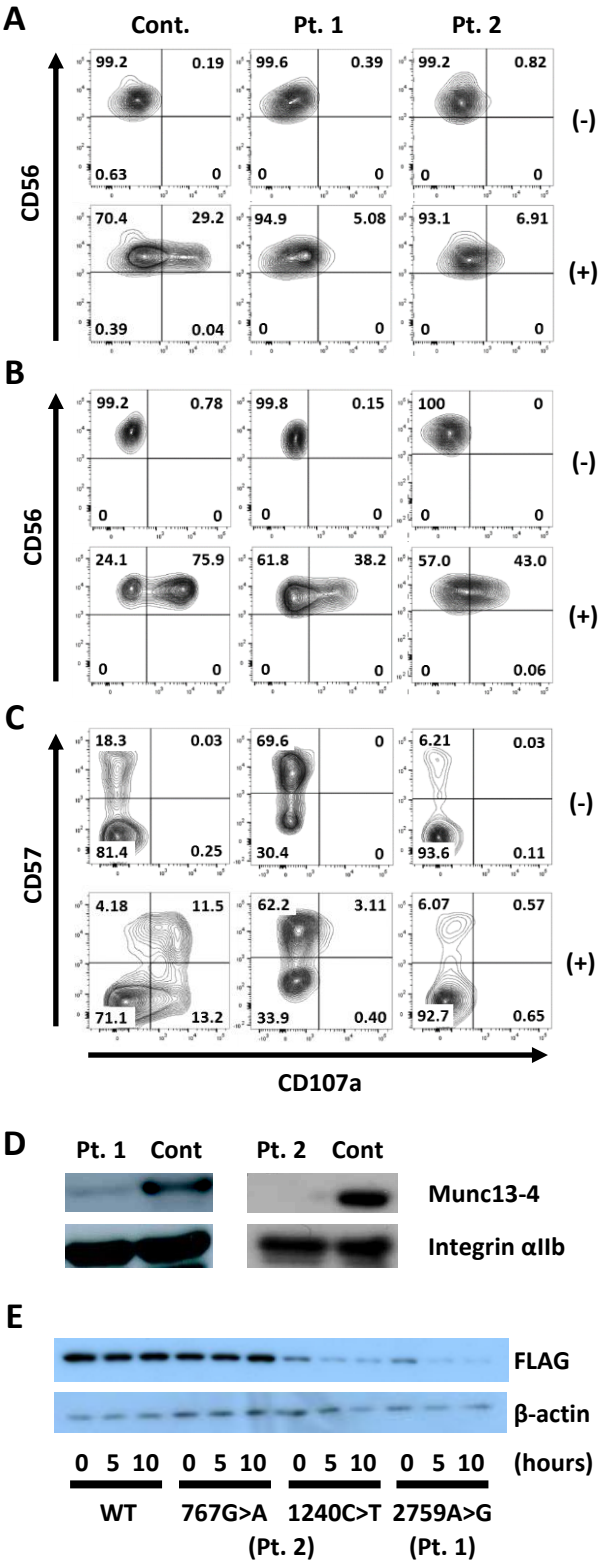
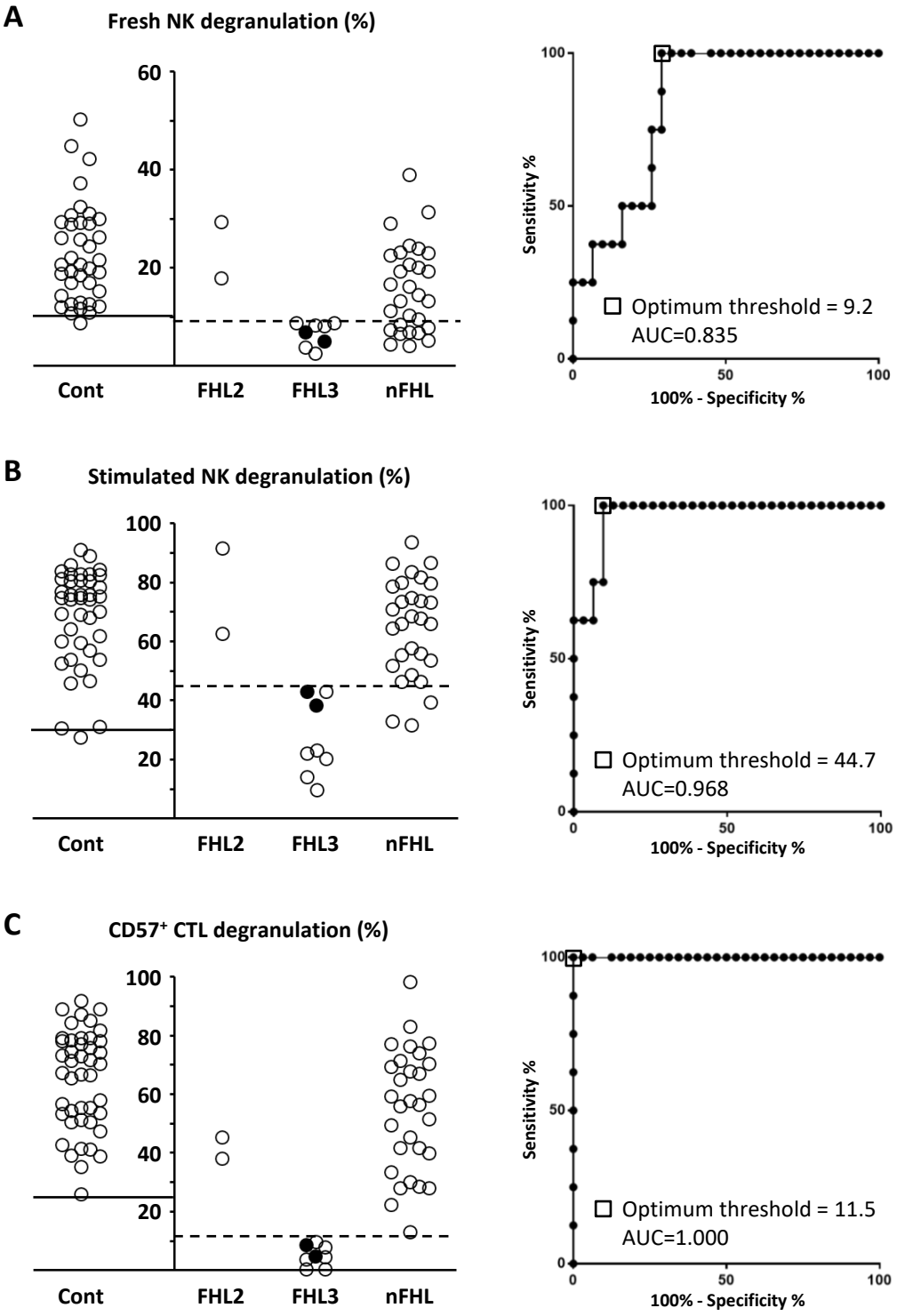

A
Cont.
Pt. 1
Pt. 2
CD56
99.2
0.19
0.63
0
99.6
0.39
0
0
99.2
0.82
0
0
(-)
70.4
29.2
0.39
0.04
94.9
5.08
0
0
93.1
6.91
0
0
(+)
B
CD56
99.2
0.78
0
0
99.8
0.15
0
0
100
0
0
0
(-)
24.1
75.9
0
0
61.8
38.2
0
0
57.0
43.0
0
0.06
(+)
C
CD57
18.3
0.03
81.4
0.25
69.6
0
30.4
0
6.21
0.03
93.6
0.11
(-)
4.18
11.5
71.1
13.2
62.2
3.11
33.9
0.40
6.07
0.57
92.7
0.65
(+)
CD107a
D
Pt. 1
Cont
Pt. 2
Cont
Munc13-4
Integrin αIIb
E
FLAG
β-actin
0 5 10
0 5 10
0 5 10
0 5 10
(hours)
WT
767G>A
(Pt. 2)
1240C>T
2759A>G
(Pt. 1)

Figure 2


A
Fresh NK degranulation (%)
60
40
20
Cont
FHL2
FHL3
nFHL
100
50
0
Sensitivity %
Optimum threshold = 9.2
AUC=0.835
0
50
100
100% - Specificity %
B
Stimulated NK degranulation (%)
100
80
60
40
20
Cont
FHL2
FHL3
nFHL
100
50
0
Sensitivity %
Optimum threshold = 44.7
AUC=0.968
0
50
100
100% - Specificity %
C
CD57+ CTL degranulation (%)
100
80
60
40
20
Cont
FHL2
FHL3
nFHL
100
50
0
Sensitivity %
Optimum threshold = 11.5
AUC=1.000
0
50
100
100% - Specificity %