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Efficacy of T cell assays for the diagnosis of primary defects in cytotoxic lymphocyte exocytosis

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Abstract:
Primary hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disorder associated with autosomal recessive variants in genes required for perforin-mediated lymphocyte cytotoxicity. A rapid diagnosis is crucial for successful treatment. Although defective cytotoxic T lymphocyte (CTL) function causes pathogenesis, quantification of natural killer (NK) cell exocytosis triggered by K562 target cells currently represents a standard diagnostic procedure for primary HLH. We have prospectively evaluated different lymphocyte exocytosis assays in 213 patients referred for evaluation for suspected HLH and related hyperinflammatory syndromes. A total of 138 patients received a molecular diagnosis consistent with primary HLH. Compared to routine K562 cell-based assays, assessment of Fc receptor-triggered NK-cell and T cell receptor-triggered CTL exocytosis displayed higher sensitivity and improved specificity for the diagnosis of primary HLH, with these assays combined providing a sensitivity of 100% and specificity of 98.3%. By comparison, NK-cell exocytosis following K562 target cell stimulation displayed a higher inter-individual variability, in part explained by differences in NK-cell differentiation or large functional reductions following shipment. We thus recommend combined analysis of T cell receptor-triggered CTL and Fc receptor-triggered NK-cell exocytosis for the diagnosis of patients with suspected familial HLH or atypical manifestations of congenital defects in lymphocyte exocytosis.

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Efficacy of T cell assays for the diagnosis of primary defects in cytotoxic lymphocyte exocytosis

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Data Sharing

Data will be shared upon e-mail to the corresponding authors.

Running title

Sensitive cytotoxic lymphocyte exocytosis assays

Keywords

Familial hemophagocytic lymphohistiocytosis, exocytosis, cytotoxic T cells, natural killer cells, flow cytometry assays, diagnosis.

Key points

1. TCR-triggered T cell and FcR-triggered NK-cell exocytosis assays are most accurate for diagnosing defective cytotoxic lymphocyte exocytosis.
2. The standard K562 cell assay has high inter-individual variability, is affected by expanded NK-cell subsets and transportation stress.

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Abstract

Primary hemophagocytic lymphohistiocytosis (HLH) is a life-threatening disorder associated with autosomal recessive variants in genes required for perforin-mediated lymphocyte cytotoxicity. A rapid diagnosis is crucial for successful treatment. Although defective cytotoxic T lymphocyte (CTL) function causes pathogenesis, quantification of natural killer (NK) cell exocytosis triggered by K562 target cells currently represents a standard diagnostic procedure for primary HLH. We have prospectively evaluated different lymphocyte exocytosis assays in 213 patients referred for evaluation for suspected HLH and related hyperinflammatory syndromes. A total of 138 patients received a molecular diagnosis consistent with primary HLH. Compared to routine K562 cell-based assays, assessment of Fc receptor-triggered NK-cell and T cell receptor-triggered CTL exocytosis displayed higher sensitivity and improved specificity for the diagnosis of primary HLH, with these assays combined providing a sensitivity of 100% and specificity of 98.3%. By comparison, NK-cell exocytosis following K562 target cell stimulation displayed a higher inter-individual variability, in part explained by differences in NK-cell differentiation or large functional reductions following shipment. We thus recommend combined analysis of T cell receptor-triggered CTL and Fc receptor-triggered NK-cell exocytosis for the diagnosis of patients with suspected familial HLH or atypical manifestations of congenital defects in lymphocyte exocytosis.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory disorder characterized by unremitting fever, splenomegaly, cytopenia, hypertriglyceridemia, hypofibrinogenemia, hemophagocytosis, as well as high serum ferritin and soluble CD25.[1-4] Early-onset, familial forms of HLH (FHL) are associated with autosomal recessive variants in *PRF1*, *UNC13D*, *STXBP2*, and *STX11*, respectively encoding perforin, Munc13-4, Munc18-2, and syntaxin-11. Perforin expression is mainly restricted to NK-cells as well as subsets of $CD8^+$ T cells, where it is contained in cytotoxic granules.[5,6] In contrast, Munc13-4, Munc18-2, and syntaxin-11 are cytosolic proteins that mediate exocytosis by hematopoietic cells. The similarities in clinical presentation of patients with perforin-, Munc13-4-, Munc18-2-, and syntaxin-11-deficiency underscore their pivotal role in facilitating target cell killing by cytotoxic T lymphocytes (CTL) and NK-cells.[7-10] Individuals with autosomal recessive variants in *RAB27A*, *LYST*, and *AP3B1* also display defective cytotoxic granule exocytosis and frequently develop HLH, with a majority manifesting characteristic hypopigmentation.[11-13] Impaired cytotoxic granule exocytosis has also been reported in patients with autosomal recessive *AP3D1*, *MADD, ORAI1*, *RHOG*, and *STIM1*.[14-18] Individuals with variants in *SH2D1A*, *XIAP*, *CD27*, or *ITK* may also manifest with HLH, but lack a gross defect in lymphocyte cytotoxicity.[19-23] Individuals diagnosed with other inborn errors of immunity (IEI) may also develop HLH without displaying defective lymphocyte cytotoxicity.[24,25] HLH may also present in the context of infections or malignancy, in individuals seemingly lacking a genetic predisposition.[26]

Primary defects in lymphocyte cytotoxicity can be rapidly fatal unless promptly treated.[27] Whereas first-line treatment involves immunosuppression, the only currently available cure is allogeneic hematopoietic stem cell transplantation. Gene therapeutic approaches are being investigated.[1,28,29] Advances in DNA sequencing technology can

facilitate rapid molecular diagnoses but may miss pathogenic non-coding genetic aberrations.[30-33] Moreover, variants of unknown significance require functional validation. Thus, functional analysis of lymphocyte cytotoxicity can provide timely and necessary answers that support vital treatment decisions.[4,8]

According to the HLH-2004 guidelines, defective NK-cell activity, as measured by the ^{51}Cr-labelled K562 target cell lysis assay, represents one criterion for the diagnosis of HLH.[1] Using radioactive isotope, which is prohibited in many laboratories, this assay requires relatively large numbers of peripheral blood mononuclear cells (PBMC). Results are also dependent on the frequency of NK-cells among PBMC, which may be influenced by inflammation. Hence, the ^{51}Cr-labelled K562 target-cell lysis assay does not unequivocally reflect the killing capacity of NK-cells.[34,35] Laboratories have therefore adopted assays quantifying intracellular perforin expression as well as induced surface expression of CD107a (also known as lysosomal associated membrane protein-1, LAMP1) in response to K562 cells for the diagnosis of patients with biallelic loss-of-function (LoF) variants in *PRF1* or *UNC13D*, *STX11*, *STXBP2*, *RAB27A*, and *LYST*, respectively.[21,36-38]

Prf1 knock-out mice have demonstrated that defective CTL activity is key to the pathogenesis of HLH, whereas defective NK-cell cytotoxicity may hamper control of adaptive immune responses and exacerbate disease.[39,40] Thus, measurements of CTL lytic activity may be of particular value for the diagnosis of primary HLH patients. Additionally, stimulation can lead to functional recovery of NK and T cells exocytosis in patients with LoF variants in *RAB27A*, *STX11*, *STXBP2*, and *LYST*.[8-10,21,41-43] Based on phenotypic characterization of peripheral blood CTL as $CD8^+CD57^+$ T cells, we previously published a protocol for sensitive evaluation of CTL exocytosis.[6] This assay aided the diagnosis of patients with *UNC13D* variants where NK-cell assays yielded contradictory results.[44] Importantly, the efficacy of different NK-cell and CTL exocytosis assays for the overall

diagnosis of patients with defects in lymphocyte cytotoxicity has not been systematically evaluated.

Here, we detail inter-individual differences in cytotoxic lymphocyte exocytosis in a large cohort of healthy individuals and prospectively evaluate the efficacy of CTL and NK-cell exocytosis assays for patients with suspected primary HLH. Quantification of CTL exocytosis triggered by T cell receptor (TCR)-engagement or NK-cell exocytosis triggered by Fc receptor CD16-engagement provided the highest levels of accuracy for the diagnosis of primary defects in cytotoxic lymphocyte exocytosis, with K562 cell-triggered exocytosis assays being less sensitive. Combined evaluation of distinct cytotoxic lymphocyte subsets provided the most reliable and accurate diagnosis of patients with defects in cytotoxic lymphocyte exocytosis.

Material and methods

Cells

This study was approved by the Stockholm Regional Ethical Review Board. Patient samples were collected between November 2011 and May 2022. Healthy individual samples were obtained from the Karolinska University Hospital Blood Bank. For methods on sequencing, please refer to the supplementary data.

Heparinized blood was received at room temperature and peripheral blood mononuclear cells extracted via density gradient centrifugation (Lymphoprep, Axis-Shield). From adults, 10 mL of blood was requested, whereas between 1-10 mL were obtained from infants and children. Live nucleated cells were counted (Muse, Cytek) and plated overnight unstimulated in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamate, all Hyclone). K562 cells (CCL243, ATCC) and P815 cells (TIB64, ATCC) were maintained in complete medium. In total, 8×10^5 PBMC were generally used for individual analyses. A detailed protocol is specified in the supplemental material.

Flow cytometric assays

Assessment of exocytosis was performed as previously described.[6] Briefly, PBMC were stimulated for three hours at with either K562 cells (CCL-243, ATCC), P815 cells (TIB-64, ATCC) coated with anti-CD16 antibody (3G8, BD Biosciences), or P815 cells coated with anti-CD3 (OKT3, BD Biosciences or S4.1, Thermo Fisher) antibody at 1:1 effector to target cell ratio. Cells were then surfaced stained with antibodies to CD3 (OKT3), CD8 (SK1), CD56 (HCD56), CD57 (HNK1), as well as an amine reactive viability dye (Thermo Fisher) and CD107a (H4A3). This test was also performed in combination with intracellular staining of signaling proteins where surface staining was followed by intracellular staining with anti-

FcεRγ (polyclonal rabbit, Upstate), anti-EAT-2 (polyclonal rabbit, Proteintech) and anti-SYK (clone 4D10.2, eBioscience) antibodies, as previously detailed.[45] Cells were acquired on a 4 laser BD Fortessa instrument. Flow cytometry data was analyzed with Flowjo (v9.9, Treestar).[21] Gates for NK-cells (Live $CD3^{-}CD56^{+}$) and CTL (Live $CD3^{+}CD8^{+}CD57^{+}$) were made based on the mock stimulated cells. These were then juxtaposed onto the various stimulated tubes where the delta value of CD107a expression was calculated by deducting the levels of background.

Statistical analysis

Statistics were generated with Prism (v7, Graphpad) as well as pROC R (v3.0.2) package for the generation of receiver operating characteristic (ROC) curves.[46] Youden's index and discriminant power (DP) was calculated as described.[47,48] Assay accuracy was defined as: (true positive + true negative)/(true positive + false positive + false negative + true negative). Unpaired t-tests are reported as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Box and whisker plots indicate quartiles (25^{th}, 50^{th}, 75^{th}), 2.5^{th} and 97.5^{th} percentiles for whiskers, and points were plotted individually for outliers. Bar graphs show lines representing SD. Specific statistics used are reported in the figure legends.

This study was approved by the Stockholm Regional Ethical Review Board.

Results

Performance of different cytotoxic lymphocyte exocytosis assays in healthy adults

In our initial description of a sensitive T cell exocytosis assay, analysis of PBMC from 14 healthy adult volunteers revealed superior responses by $CD8^+CD57^{dim}$ and $CD8^+CD57^{bright}$ T cells following TCR-engagement as compared to other T cell as well as NK-cell subsets following antibody-mediated engagement of CD16 or K562 cell stimulation.[6] To validate these findings from a large number of healthy adults, we assessed cytotoxic lymphocyte exocytosis in 198 healthy adult volunteers (Figure 1A). Substantiating previous findings, assessment of CD107a surface expression in response to engagement of the TCR on $CD8^+CD57^{dim}$ and $CD8^+CD57^{bright}$ T cells or CD16 on NK-cells using anti-CD3 or anti-CD16 mAbs, respectively, demonstrated the strongest responses with a coefficient of variation (CV) of 0.22, 0.14 and 0.20, respectively. By comparison, $CD3^-CD56^{dim}$ NK-cell responses to K562 cells and $CD8^+CD57^-$ T cell responses to TCR engagement were weaker and displayed greater donor variability (CV of 0.33 and 0.33, respectively). In summary, the exocytic response of cytotoxic $CD8^+CD57^+$ T cells upon TCR engagement or $CD3^-CD56^{dim}$ NK-cells in response to CD16 engagement was the most robust.

Inter-assay reproducibility of cytotoxic lymphocyte exocytosis assays

Inter-assay variability may represent a confounding factor in the diagnosis of HLH. We therefore repeatedly tested the same two donors over 3 months, examining exocytosis by $CD8^+CD57^+$ T cells in response to TCR engagement and $CD3^-CD56^{dim}$ NK-cells in response to CD16 engagement or K562 cell stimulation. PBMC cryopreserved in multiple vials were thawed and run at different time points. Results were comparable throughout the period (Figure 1B, C). Although numerical values for exocytosis varied more for TCR engagement

or CD16 engagement (Figure 1B, C, Supplemental Table S1), the greatest relative variability was observed for $CD3^{-}CD56^{dim}$ NK-cell responses to K562 cells (Figure 1D-F). Nonetheless, the overall high inter-assay consistency provided confidence when comparing data accumulated from independent experiments collected over several years.

Variables associated with low cytotoxic lymphocyte exocytosis in healthy adults

With available data on gender, age, cytomegalovirus (CMV) serostatus, cell numbers and differentiation of the 198 healthy adult volunteers for which cytotoxic lymphocyte function was examined, additional factors influencing the magnitude of response in distinct cell subsets could be interrogated. When stratified by gender or age, no statistical difference was found among the three exocytosis assays (Supplemental Figure 1).

Forming latent infection, CMV imprints human immune cell constitution and function, foremost promoting effector $CD8^{+}$ T cell differentiation, elevating serum IL-6 and IL-10 and influencing responses to these cytokines.[49,50] Notably, $CD8^{+}CD57^{+}$ T cell exocytosis upon TCR engagement was somewhat higher in CMV seropositive individuals, whereas NK-cell responses to CD16 engagement or K562 cells were not correlated to CMV serostatus (Figure 2A).

CMV infection is associated with clonal expansions of long-lived, adaptive NK-cells that variegatedly lack expression of certain cytosolic signaling proteins.[45,51-54] Such adaptive NK-cells typically constitute a small subset of $CD56^{dim}$ NK-cells in most CMV seropositive individuals but may in some individuals dominate the NK-cell repertoire. As adaptive NK-cells display altered signaling and target cell recognition, we hypothesized that variability in NK-cell responses to K562 cells may be associated with adaptive NK-cell expansions. Indeed, NK-cells lacking FcεRγ or SYK expression displayed less exocytosis in response to

K562 cell stimulation, whereas responses to engagement of CD16 were maintained (Figure 2B). Among the 198 healthy individuals examined, expansions of NK-cells lacking FcεRγ, EAT-2, or SYK expression did not affect $CD3^{-}CD56^{dim}$ NK-cells responses to CD16 engagement (Figure 2C). However, highly reduced FcεRγ or SYK expression correlated with low $CD3^{-}CD56^{dim}$ NK-cell exocytosis upon K562 cell stimulation (Figure 2D). A few healthy individuals with large adaptive $CD3^{-}CD56^{dim}Fc\varepsilon R\gamma^{-}$ NK-cell subsets displayed K562 cell-induced exocytosis below the 10% threshold that has been deemed abnormal (Figure 2D).[21] Lack of FcεRγ, EAT-2 and SYK expression is stochastically interlinked.[45] Boolean gating of adaptive NK-cell subsets demonstrated that lack of FcεRγ, but not EAT-2 or SYK specifically correlated with reduced NK-cell exocytosis in response to K562 cell stimulation (Figure 2E). Thus, NK-cell responses to K562 cells were diminished in individuals with a high proportion of adaptive NK-cells lacking expression of FcεRγ.

Finally, we compared TCR-triggered exocytosis in $CD8^{+}CD57^{+}$ T cells to CD16-triggered exocytosis in $CD3^{-}CD56^{dim}$ NK-cells. Notably, a few donors displayed low responses to both these stimuli, additionally displayed low $CD3^{-}CD56^{dim}$ NK-cell exocytosis in response to K562 cells (Figure 2F, G), but were distinct from low responders with respect to K562 cell recognition explained by epigenetic regulation of signaling protein expression (Figure 2H). Thus, combined assays may discriminate healthy adult individuals with overall mild impairments in cytotoxic lymphocyte exocytosis.

Prospective evaluation of cytotoxic lymphocyte exocytosis assays in hyperinflammatory patients

To gain insights to the efficacy of different cytotoxic lymphocyte exocytosis assays for the diagnosis of FHL, we prospectively evaluated cohorts of patients fulfilling HLH criteria,

carrying HLH-associated LoF variants, or manifesting related hyperinflammatory disorders. Patients that exhibited reduced exocytosis according to established guidelines, or with at least 5 out of 8 HLH criteria fulfilled, or with a family history of HLH were sequenced for HLH-associated genes.[21] Furthermore, in patients with hypopigmentation, *LYST*, *AP3B1*, and *RAB27A* were sequenced.

Notably, a total of 92 patients analyzed harbored biallelic *UNC13D*, *STX11*, *STXBP2*, *LYST*, *RAB27A*, *AP3B1*, or *RHOG* LoF variants (termed EXO). Moreover, a total of 58 patients with biallelic or hemizygous *PRF1*, *SH2D1A*, *XIAP*, *CD27*, *GATA2*, *MAGT1*, *ZNFX1*, *CYBA*, or *ITK* LoF variants were analyzed (termed IEI). Thirty-three patients fulfilling HLH criteria but lacking pathogenic variants in genes associated with familial HLH or IEI and 30 patients diagnosed with macrophage-activation syndrome (MAS) or systemic-onset juvenile idiopathic arthritis (SoJIA) were also analyzed (termed HYPINF). These results were compared to those from 84 unrelated adult transport control samples or 198 healthy adult controls derived from the local blood bank. Three exocytosis stimulations were performed, namely, K562 cell-triggered NK-cell exocytosis, Fc receptor-triggered anti-CD16 antibody $CD3^{-}CD56^{dim}$ NK-cell exocytosis, and TCR-triggered anti-CD3 antibody CTL exocytosis. Overall, we compared five sample groups and three stimulations (Table 1, Figure 3).

Patients in the EXO group displayed reduced exocytosis upon stimulation of CTL and NK-cells as compared to IEI or HYPINF patients, healthy adults, or transport controls (Figure 3A-B). Patients diagnosed with FHL3 (*UNC13D*), FHL4 (*STX11*), FHL5 (*STXBP2*), CHS (*LYST*) and GS2 (*RAB27A*) displayed defective exocytosis in all assays (Figure 3D), whereas patients diagnosed with FHL2 (*PRF1*), XLP1 (*SH2D1A*), XLP2 (*XIAP*) or other IEI generally displayed exocytosis almost at the level of healthy adults controls (Figure 3E). Notably, trendlines for exocytosis in IEI or HYPINF patients plotted versus age at analysis

showed similar levels of exocytosis in pediatric subjects to that of healthy adult control samples (Supplemental Figure 2A).

Sensitivity and specificity of individual cytotoxic lymphocyte exocytosis assays

Our exocytosis data was used to plot ROC curves (Figure 4A-D, Supplemental Figure 2B-E). The area under the curve (AUC) was larger for anti-CD3 CTL and anti-CD16 NK-cell exocytosis assays. The K562 cell exocytosis assay returned 93.1% sensitivity and 87.0% specificity at a 5.3% exocytosis cut-off. By comparison, the anti-CD16 stimulation of NK-cells returned 96.6% sensitivity and 92.4% specificity at a 12.5% exocytosis, and the anti-CD3 stimulation of CTL was 94.8% sensitivity and 92.4% specificity at 23.5% exocytosis. The accuracy of the anti-CD3 and anti-CD16 assays was 93.3% and 98.7%, respectively, compared to 89.3% for the K562 cell assay (Table 2). The discriminant power (DP) and Youden's index showed a similar trend with K562 cell-induced NK-cell exocytosis having poorer results compared to the anti-CD3 CTL or anti-CD16 NK-cell exocytosis assay (Table 2).

Exocytosis values for the EXO, IEI and HYPINF patient groups were plotted for the three different pair-wise comparisons (Figure 4E-G). Overall, the correlation between the exocytosis values obtained in the different assays was high, with a few exceptions of patients with low NK-cell exocytosis induced by K562 cells yet with higher exocytosis induced by anti-CD16 NK-cell or anti-CD3 CTL assays.

In summary, the anti-CD3 CTL and anti-CD16 NK assays were more accurate than the current standard K562 cell-triggered NK-cell assay in predicting primary genetic defects affecting cytotoxic lymphocyte exocytosis.

Predictive power of combined analyses for the diagnosis of defective cytotoxic lymphocyte exocytosis

Combining analysis of distinct cell types could potentially increase the accuracy for detecting primary defects in lymphocyte cytotoxicity. We therefore performed combined analyses of the sensitivity and specificity values of two assays, *i.e.* K562 cells with anti-CD16 NK-cell assays, anti-CD16 NK-cell with anti-CD3 CTL assays, or, K562 cell NK-cell with anti-CD3 CTL assays. To have both true negative and true positive samples, the EXO and IEI groups were pooled. Taking cut-off values of 5.3% for K562 cell-triggered NK-cell exocytosis, 12.5% for anti-CD16 NK-cell, and 23.5% for anti-CD3 CTL assays, the results were evaluated with "AND" or "OR" Boolean functions. Utilizing "AND" did not improve the accuracy but anti-CD16 NK-cell OR anti-CD3 CTL assays returned 100% sensitivity and 98.3% specificity for a combined 99.3% accuracy (Table 3). Thus, combined analyses can enhance the diagnostic accuracy of functional diagnostic approaches.

Robustness of exocytosis assays in relation to transport duration

Exocytosis testing is typically analyzed in larger experienced centers, necessitating sample transport. Some clinical laboratories have strict cut off time points of <24 hours transport, but this is not always feasible from remote locations. Retrospectively, exocytosis assays from IEI patients were found impartial to shipment duration up to 48 hours (Supplemental Figure 3A). Samples from healthy unrelated controls arriving <48 hours generally did not show any bias towards lower exocytosis results (Supplemental Figure 3B). Familial controls (Supplemental Figure 3D) show slightly poorer exocytosis at the >48 hours timepoint. When grouped by time of arrival (Supplemental Figure 3C, E), a difference in function was observed when comparing samples arriving <24 hours to those arriving >48 hours. Notably, anti-CD16-

triggered NK-cell exocytosis appeared the most time stable test. We further investigated stability on blood samples from 10 healthy adult volunteers left for 1, 24, 48, and 72 hours before PBMC isolation. A significant reduction of exocytosis potential with all three stimulations was observed within 24 hours post venipuncture (Supplemental Figure 3F). For K562 cell-triggered NK-cell exocytosis, the responses continuously dropped up to 48 hours while anti-CD16-triggered assays remained stable during that time followed by a drop in function at >48 hours. The anti-CD3 CTL assay was the most robust with no statistically significant reduction in function even in samples stored >48 hours. The contrast in results between shipped and laboratory controls highlighted the difficulty in controlling for transportation stress as it is unique for each shipment and may include not just time but also variables such as temperature and agitation. In summary, as the anti-CD3 CTL exocytosis assay has inherently high signal to noise ratio and assay sturdiness, although not ideal, evaluation of cytotoxic lymphocyte function is possible with samples arriving after more than 48 hours.

Discussion

We previously optimized an assay to evaluate exocytosis by freshly isolated human CTL, without prior stimulation or prolonged incubation.[6] Here, we assessed the performance of different NK-cell and T cell exocytosis assays in a large cohort of healthy individuals and prospectively evaluated patients with suspected primary HLH collected over 10 years. Our results demonstrate that assessment of $CD8^{+}CD57^{+}$ T cells exocytosis in response to TCR-engagement and $CD3^{-}CD56^{dim}$ NK-cell exocytosis in response to CD16-engagement provided the highest diagnostic sensitivity. Importantly, combined analysis provided the highest diagnostic accuracy and safeguarded results in challenging patients where ambiguous results, long or suboptimal shipment, or low blood CTL or NK-cell frequencies might lead to assay failure.

Evaluation of ^{51}Cr-labelled K562 target-cell lysis has historically represented a gold-standard for evaluation of cytotoxic lymphocyte activity and diagnosis of primary HLH.[55,56] However, this test carries a number of drawbacks which may yield false positive results.[1] Hence, quantification of surface CD107a up-regulation on NK-cells after K562 cell stimulation was proposed as a diagnostic assay for patients with defective cytotoxic lymphocyte exocytosis.[21,57] Initially identified as an activation-induced surface protein on PBMC subsets, CD107a surface expression could play a role in self-protection against released cytolytic proteins and has been used to quantify cytotoxic T cell, NK-cell, basophil, and mast cell exocytosis.[58-64] The K562 cell-triggered NK-cell exocytosis represents a safe, rapid, easily-performed, and robust assay with good sensitivity and specificity,[21,37,65] but our efforts demonstrate that even higher diagnostic accuracy can be achieved with additional stimulations.

Primary HLH is commonly viewed as a T cell-driven pathological response to infection.[39] The main aim of our study was to assess the efficacy of T cell exocytosis assay for the diagnosis of congenital defects in lymphocyte exocytosis. The assessment of T cell blasts exocytosis was previously tested, but may display a low signal-to-noise ratio especially with milder defects.[21,66] Confirming a pilot study, our data from 198 healthy adult donors revealed uniformly strong exocytic responses of CTL with relatively low inter-individual variability.[6] In comparison to assays evaluating NK and T cell exocytosis in response to engagement of CD16 or the TCR complex, respectively, the K562-NK-cell test displayed lower signal and higher inter-individual variability. Notably, with our modification of the exocytosis assay examining three read-outs of cytotoxic lymphocyte exocytosis, the cellular input is still lower than that typically used for ^{51}Cr-release assays. Hence, flow cytometric exocytosis assays are more sensitive and require lower numbers of PBMC than ^{51}Cr-release assays, potentially facilitating measurements in prenatal infants or severely lymphopenic patients. CTL assays can facilitate assessment of patients with few NK-cells, which we find represents an issue in approximately 3% of pediatric and 6% of adolescent and adult patient samples.

Low NK-cell reactivity towards K562 cells among healthy individuals correlated with outliers harboring high proportions of CMV-associated adaptive NK-cells lacking FcεRγ expression. Our results implicate FcεRγ-associated activating NKp30 and NKp46 receptors in recognition of K562 cells. Congruently, the NKp30-ligand B7-H6 is highly expressed on K562 cells and antibody-mediated NKp30 blockade inhibits NK-cell recognition of K562 cells.[67] Our results demonstrate that a high proportion of FcεRγ-negative adaptive NK-cells can negatively skew the overall NK-cell response to K562 cell stimulation. Although adaptive NK-cell expansions are associated with CMV seropositivity, in a European population, only 15% of healthy blood volunteers manifested sizeable adaptive NK-cell

expansions constituting more than 30% of the total CD56^{dim} NK-cell population.[45] CMV seropositivity is thus a poor indicator of adaptive NK-cell expansions. However, in a Sub-Saharan population, adaptive NK-cell expansions were much more prevalent and constituted more than 50% of the total CD56^{dim} NK-cell population in a majority of subjects.[68] Inflammation can promote adaptive NK-cell expansions.[69,70] Notably, patients with *GATA2* variants occasionally present with HLH, and can have extremely high frequencies of adaptive NK-cells.[53,71,72] As such, diminished NK-cell exocytosis in response to K562 cell stimulation could reflect epigenetic cell differentiation processes rather than primary HLH and be especially prevalent in individuals with severe inflammation or underlying primary genetic defects. Quantification of NK or CTL exocytosis triggered by anti-CD16 or anti-CD3, respectively, can thus safeguard K562 cell-based analyses.

Prospectively, we evaluated 213 patients with suspected primary HLH. Patients with biallelic *UNC13D*, *STX11*, *STXBP2*, *LYST*, *RAB27A*, *AP3B1*, and *RHOG* variants consistently displayed strongly impaired CTL exocytosis as compared to patients with *PRF1*, *SH2D1A*, *XIAP*, *CD27*, *GATA2*, *MAGT1*, *ZNFX1*, *CYBA*, or *ITK* variants, which have all been associated with HLH or low NK-cell numbers. Of note, healthy adult controls do not take into account several factors that might influence cellular analyses, including differences in age, time from sampling to analysis, medication/therapy, and blood inflammatory status. Thus, samples from patients diagnosed with other pathogenic HLH variants that do not impair exocytosis, secondary HLH, or other inflammatory syndromes represent better groups for comparison to patients with primary defects in cytotoxic lymphocyte exocytosis. Moreover, the interpretation of patient results is normally performed by juxtaposing a reference range obtained from a group of healthy adult donors. This study instead focuses on estimating NK and CTL exocytosis ranges of various genetically confirmed groups. Our patient data demonstrated superior sensitivity of the anti-CD3 CTL and anti-CD16 NK-cell

exocytosis assays for identification of patients with biallelic LoF variants in genes required for cytotoxic lymphocyte exocytosis as compared to the established K562 cell stimulation assay, providing a high discriminant power and Youden's index, signifying the assays' ability to discriminate between the two populations and avoid false positives and negatives, respectively.

Most cases of primary HLH are acute and require urgent diagnosis. Offering more confidence in findings, running the anti-CD3 CTL and anti-CD16 NK-cell exocytosis assays side-by-side increased accuracy and provided up to 100% sensitivity and 98% specificity. Infants below three months old normally have low numbers of $CD57^+$ T cells and it is not uncommon for patients to have too few NK-cells for evaluation, suggesting a place for testing expanded T cell exocytosis.[18,21,73,74] Independent assays also lessen the risk of human error leading to assay failure. Thus, running multiple assays increases the probability that at least one result is informative, avoiding delays and lowering the risk of misdiagnosis. Importantly, combining two or more assays also strengthens the overall diagnostic prediction. Generally, implementing changes in laboratory assays in a clinical setting can be costly in terms of efforts to fulfill national regulatory requirements, validate assays and establish reference ranges. In terms of operating time and costs for implementing Fc receptor stimulation of NK-cells and TCR-stimulation of CTLs, use of multichannel pipettes and flow cytometers with plate-readers should imply no drastic increase in operator time to run assays while reagent costs are deemed to double due to an increase from two to four wells for each tested individual.

While we report specific cut-off percentages for the three exocytosis assays, results will vary among laboratories. Moreover, our values were estimated by comparing controls and confirmed patient data, with the latter being quite rare and thus challenging for smaller laboratories to clinically validate. Determination of specific normal values are necessary for

each laboratory and protocol. Results showing defective/absent exocytosis below cut-off should be repeated to confirm while those with reduced but not defective exocytosis should also be repeated and sequenced as data here suggest some patients with hypomorphic variants in genes required for exocytosis have lowered but not absent exocytosis. Here, the K562 cell exocytosis cut-off of 5.3% is in line with the previously stipulated 5%,[21] even with protocol changes and extension from 2 to 3 hours incubation. Importantly, the CTL exocytosis assay is also more robust as compared to the K562 cell assay when dealing with suboptimal samples, due to long or harsh transportation.[31] Large numbers of familial and healthy unrelated controls sent together with patient samples were minimally affected by transport times up to 48 hours, indicating that successful diagnosis of samples from farther locales or long shipping duration is feasible if well controlled.

We have demonstrated the accuracy of various exocytosis assays in diagnosing different primary genetic defects causative of defective exocytosis. We recommend laboratories run a combination of two exocytosis assays with other panels to investigate levels of perforin, SAP, XIAP, CD27, NKT cell, and TCRα/β- double negative T cells to encompass a wider range of primary genetic defects linked to HLH as illustrated in our suggested laboratory diagnosis algorithm (Figure 5).[22,23,75-83] We have previously reported how IL-2 stimulation can discriminate certain forms of FHL.[6,8,21,41-43] In the present study we focused analyses on freshly isolated samples where functional assays can promptly identify patients with defective exocytosis and potentially direct treatment. With respect to molecularly categorizing patients, we advocate that genetic tests are a more reliable way stratify FHL patients and gain important genotype-functional correlations.

In conclusion, screening for anti-CD3 antibody induced exocytosis of CTL and anti-CD16 antibody induced exocytosis of NK-cells are more accurate than the current standard K562 cell stimulation for the diagnosis of congenital defects in lymphocyte exocytosis. The

data presented here thus suggests a change in the HLH-2004 criteria from low or absent NK-cell activity to low NK-cell or CTL exocytosis, except for FHL2/perforin-deficiency. Performing two different exocytosis stimulations simultaneously gives high accuracy and acts as confirmatory assays. Such assays with increased sensitivity can aid the understanding of immunodeficiencies that may partially impair exocytosis.

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Conflict of interest

The authors declare no competing interests.

Authorship

S.C.C.C. designed the study, performed exocytosis assays, evaluated the data, and wrote the manuscript. B.T. and L.E.C. performed sequencing, evaluated the data, and performed ROC analyses. H.S. performed exocytosis assays with EAT-2 and and FcεRγ staining. J.T. partially performed statistical analysis. T.M.C, J.N.-Z., T.S., and S.W. performed exocytosis assays. M.M. performed sequencing and evaluated the data. K.M., W.A.-H., H.H.A., F.B.B., M.Y.C., O.D., T.A., M.I., I.M., M.S., E.U., S.U., W.I., K.K., K.C.G., S.E. cared for patients and/or provided material. All pHLH collaborators cared for patients and provided clinical information. H.-G.L., M.N., A.H., and J.-I.H. revised the manuscript. Y.T.B. designed the study, evaluated the data and wrote the manuscript.

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Table 1. **Categorization and genetic characterization of samples.**

Category	Subgroup	Number of individuals	Subtotal
EXO	*UNC13D* (FHL3)	35	
	STX11 (FHL4)	12	
	STXBP2 (FHL5)	13	
	LYST (CHS)	19	
	RAB27A (GS2)	10	
	AP3B1 (HPS2)	2	
	RHOG	1	**92**
IEI (non-EXO)	*PRF1* (FHL2)	30	
	SH2D1A (XLP1)	8	
	XIAP (XLP2)	8	
	CD27	3	
	GATA2	3	
	MAGT1 (XMEN)	2	
	ZNFX1	2	
	CYBA	1	
	ITK	1	**58**
HYPINF	Secondary HLH	33	
	soJIA/MAS	30	**63**
HD-CTRL	Healthy donors	198	**198**
TRP-CTRL	Transport controls	84	**84**
Grand total			**495**

Number and categories of patients included in this study by their respective groups retrospectively compiled from one center over 10.5 years. EXO: individuals with NK/CTL exocytosis defect, IEI: individuals with other inborn errors of immunity excluding EXO, HYPINF: individuals with a hyperinflammatory syndrome fulfilling HLH criteria; HD-CTRL: healthy adult volunteers; and (TRP-CTRL) healthy adult transport controls. XLP: X-linked lymphoproliferative disease, XMEN: X-linked MAGT1 deficiency with increased susceptibility to EBV infection and N-linked glycosylation defect.

Table 2. Statistics on specific sample group comparisons.

	Stimulation	Cut-off CD107a$^+$ (%)	Sensitivity	Specificity	Youden	DP	Accuracy
EXO vs IEI	*K562*	5.3	93.1	87.0	0.81	1.08	89.3
	anti-CD16	12.5	96.6	92.4	0.89	1.40	98.7
	anti-CD3	23.5	94.8	92.4	0.88	1.29	93.3
EXO vs HYPINF	*K562*	8.0	81.0	95.7	0.78	1.09	89.7
	anti-CD16	14.3	98.4	94.6	0.93	1.67	96.1
	anti-CD3	21.2	98.4	91.3	0.90	1.55	94.2
EXO vs HD-CTRL	*K562*	9.9	97.8	98.0	0.96	1.84	97.9
	anti-CD16	26.3	98.9	99.0	0.98	2.18	99.0
	anti-CD3	40.9	98.9	99.5	0.98	2.34	99.0
EXO vs TRP-CTRL	*K562*	7.8	91.7	95.7	0.88	1.32	93.2
	anti-CD16	15.2	98.8	94.6	0.93	1.74	96.6
	anti-CD3	21.3	95.2	91.3	0.87	1.28	93.2

Optimal cut-off for exocytosis assay (CD107a$^+$ %), sensitivity, and specificity obtained through ROC plots comparing three different exocytosis assays for patients with variants associated with defective exocytosis (EXO) versus four other control groups [other inborn errors of immunity (IEI), other hyperinflammatory diseases (HYPINF), 198 healthy adult volunteers (HD-CTRL), and healthy adult transport controls (TRP-CTRL)]. Youden's Index, discriminant power (DP), and accuracy were calculated based on the obtained values.

Table 3. Increased accuracy provided by combined assessment of exocytosis in cytotoxic T cell and NK-cell subsets.

		Sensitivity	Specificity	Youden	Accuracy
K562 with anti-CD16					
	OR	100.0	98.3	>10	99.3
	AND	87.0	91.4	1.02	88.7
K562 with anti-CD3					
	OR	97.8	98.3	1.88	98.0
	AND	81.5	91.4	0.92	85.3
anti-CD16 with anti-CD3					
	OR	100.0	98.3	>10	99.3
	AND	92.4	93.1	1.22	92.7

Combining two different exocytosis assays lead to improved sensitivity or specificity. Displayed are calculated sensitivity, specificity, Youden's index, and accuracy after combining results from two exocytosis assays with "AND" or "OR" Boolean gating.

Figure legends

Figure 1. Analysis of experimental inter-assay variability in evaluation of cytotoxic lymphocyte exocytosis. (A) PBMC from 198 healthy adult volunteers were incubated for 3 hours in medium or with target cells and mAbs as indicated. Induced CD107a surface expression (ΔCD107a) values for distinct cytotoxic lymphocyte subsets and stimulations, as indicated, in 198 healthy adult volunteers. Graph depicts mean values with bars indicating SD. **(B-F)** Compiled induced CD107a surface expression values for distinct cytotoxic lymphocyte subsets and stimulations, as indicated, from two healthy adult volunteers run 17 (Donor 1) and 23 times (Donor 2) using multiple frozen PBMC vials thawed over a period of three months. Samples were run using the same exocytosis protocol. Shown are the ΔCD107a for $CD3^{-}CD56^{dim}$ NK-cells after K562 or anti-CD16 stimulation, and $CD8^{+}CD57^{+}$ T cells (CTL) after anti-CD3 stimulation. Results plotted against time for **(B)** Donor 1 and **(C)** Donor 2, displaying results of the three different exocytosis stimulations. The same data was normalized by dividing each value by respective mean and plotted against time for **(D)** Donor 1 and **(E)** Donor 2, demonstrating the range of deviation for each assay and donor. **(F)** Accumulated normalized data for each assay. Bars indicate SD.

Figure 2. **Analysis of the variability in cytotoxic lymphocyte exocytosis in healthy adults. (A-H)** PBMC from 198 healthy adult volunteers were incubated for 3 hours in medium or with target cells and mAbs as indicated. **(A)** Graph displays levels of exocytosis in $CD8^{+}CD57^{+}$ T cells or $CD3^{-}CD56^{dim}$ NK-cells stratified according to CMV serostatus. Box and whiskers display quartiles, limits, as well as outliers, with plus signs representing means. Pairs statistically different by Student's t-test are indicated. **(B)** Flow cytometry plots show $CD3^{-}CD56^{dim}$ NK-cell CD107a surface expression in relation to intracellular expression of

FcεRγ or EAT-2, following indicated stimulations. The percentages of cells in each quadrant are indicated. Only 5.4% of FcεRγ$^-$ NK-cells exocytosed following K562 stimulation as compared to 21.7% of FcεRγ$^+$ NK-cells. Similarly, 6.7% of EAT-2$^-$ NK-cells exocytosed when stimulated with K562 as compared to 25% of EAT-2$^+$ NK-cells. (**C, D**) Graphs displaying the relationship between CD3$^-$CD56^{dim} NK-cell induced CD107a surface expression and frequencies of cells lacking expression of FcεRγ, EAT-2 or SYK following stimulation with **(C)** P815 cells added anti-CD16 mAb or **(D)** K562 cells. Diagonal lines represent linear regression and with 95% confidence intervals in bold while the dashed horizontal line indicates the cut-off percentage deemed abnormal in regards to K562 cell-induced NK-cells exocytosis. **(E)** The graph depicts frequencies of CD107a-expressing NK-cells according to stimulation and expression of FcεRγ, SYK, and EAT-2 in CD3$^-$CD56^{dim} NK-cell subsets, with bars representing SD. **(F-H)** Graphs correlating exocytic responses in cytotoxic lymphocyte subsets in healthy individuals, as specified. Donors with low CD107a response after K562 stimulation and expanded FcεRγ$^-$ NK-cell populations are highlighted with red and orange dots, while donors with low CD107a responses to anti-CD3 and anti-CD16 are highlighted with blue dots.

Figure 3. Exocytosis is defective in cytotoxic lymphocytes of FHL3-5, CHS, and GS2 patients. Percentages of CD8$^+$CD57$^+$ T cell **(A)** or CD3$^-$CD56^{dim} NK-cells **(B, C)** cells expressing surface CD107a after incubation with **(A)** P815 cells with anti-CD3 antibody, **(B)** P815 cells with anti-CD16 antibody, or **(C)** K562 cells, shown for five different groups, as indicated. Values represent the difference in CD107a levels of stimulated against control unstimulated cells. Boxes display quartiles and whiskers display 2.5 and 97.5 percentiles, as well as outliers. Student's t-test comparing EXO against the other groups, **** p<0.0001. (**D-E**) Exocytosis data of specific patient categories from the 'impaired exocytosis' and 'other

IEI patients' groups are shown individually. Each individual patient value is represented by a symbol, with violin plots indicating quartiles. Colored markings depict normal ranges from 198 healthy adults with boxes displaying quartiles and whiskers displaying 2.5 and 97.5 percentiles. **(E)** Student's t-test comparing patients to 198 heathy adult controls; *n.s.* not significant, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Figure 4. Combination receiver operating characteristic (ROC) curves from this study. (**A-D**) Combined ROC curves for different exocytosis stimulations (K562 cell stimulation for NK-cells, anti-CD16 antibody for NK-cells, and anti-CD3 antibody for CTL) when comparing 92 impaired exocytosis (EXO) patients against four other control groups consisting of (**A**) 58 other inborn errors of immunity (IEI patients), (**B**) 63 hyperinflammatory (HYPINF) patients, (**C**) 198 healthy adults controls, or (**D**) 84 healthy adults transport controls. Cut-off values with sensitivity and specificity are indicated in brackets for each comparison. (**E-F**) Plots show exocytosis values for different cytotoxic lymphocyte subsets and stimulations, as indicated. Each circle represents an individual patient color coded according to indicated key.

Figure 5. Proposed laboratory diagnostic algorithm for patients presenting with HLH. As the spectrum and our understanding of primary diseases linked to HLH grows, laboratories would need to constantly update themselves with the latest diagnostic assays. It is recommended that individual laboratories conduct tests to determine local cutoff values for the respective assays. Functional XIAP assay[80] should be considered in both males and females in light of recent findings,[81,82] reduced iNKT numbers a hallmark of ITK[23,78] but cautionary for SAP deficiency with highly variable levels in young children.[76,83] Elevated α/β-DNT cells raises suspicion of ALPS.[77] The total absence CD27 in all lymphocyte

subgroups is indicative of its deficiency[22,79] while absence of CD27 specifically in the B cell compartment is characteristic of SAP deficiency[75]. While the functional XIAP and NK/CTL exocytosis assays should be performed on heparinized blood, if EDTA blood is available, it is preferable for phenotyping tests (perforin, SAP, XIAP, CD27, α/β-DNT) as it is more stable with transport, can be used for DNA extraction, and plasma collected for analyzing cytokine levels. XIAP: X-linked inhibitor of apoptosis protein, iNKT: invariant NKT, ITK: Interleukin-2-Inducible T-Cell Kinase, ALPS: Autoimmune lymphoproliferative syndrome, SAP: SLAM–associating protein, DNT: double negative T, EDTA: Ethylenediaminetetraacetic Acid, DNA: Deoxyribonucleic acid.

Figure 1

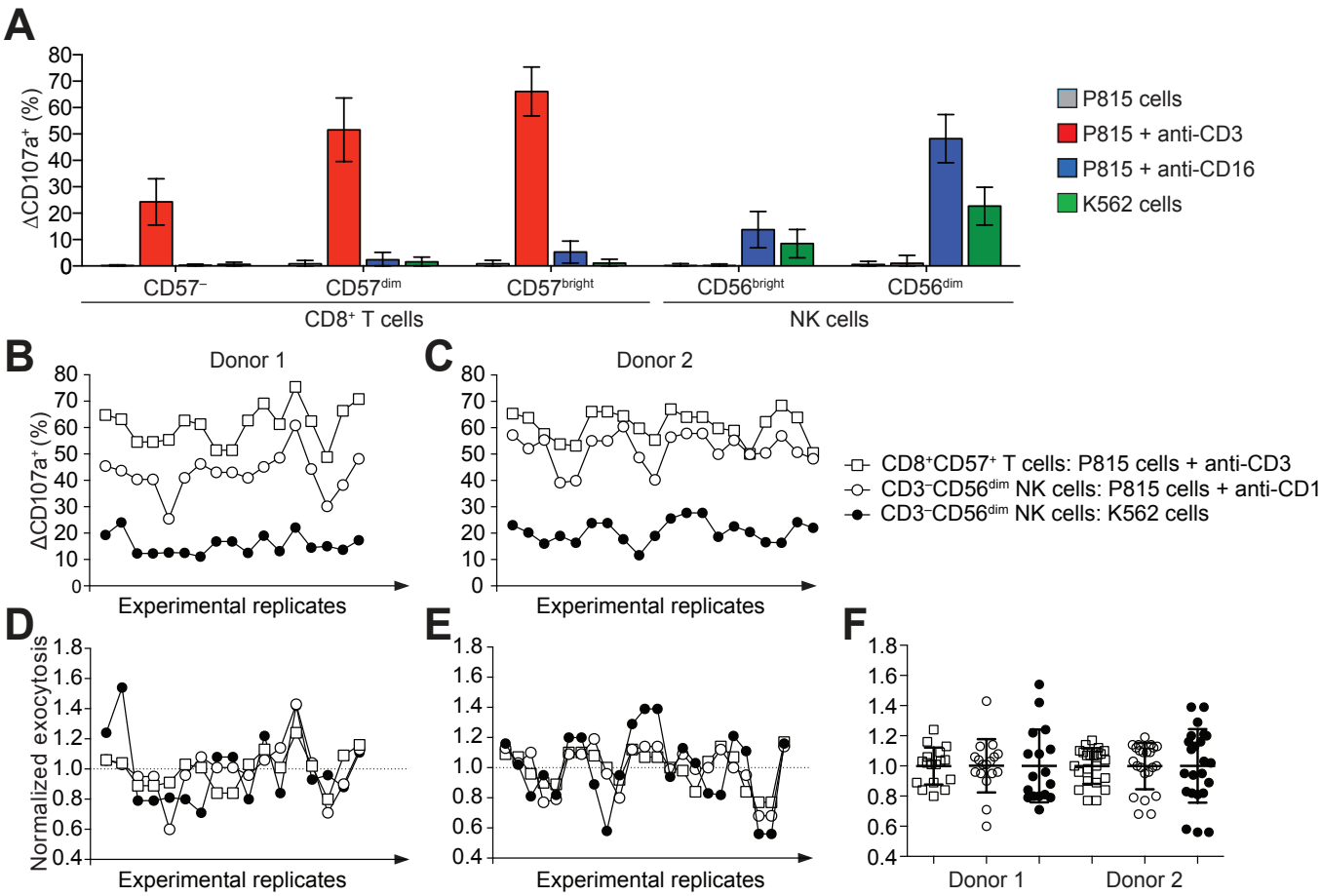


Figure 1. Chiang *et al*.

Figure 2

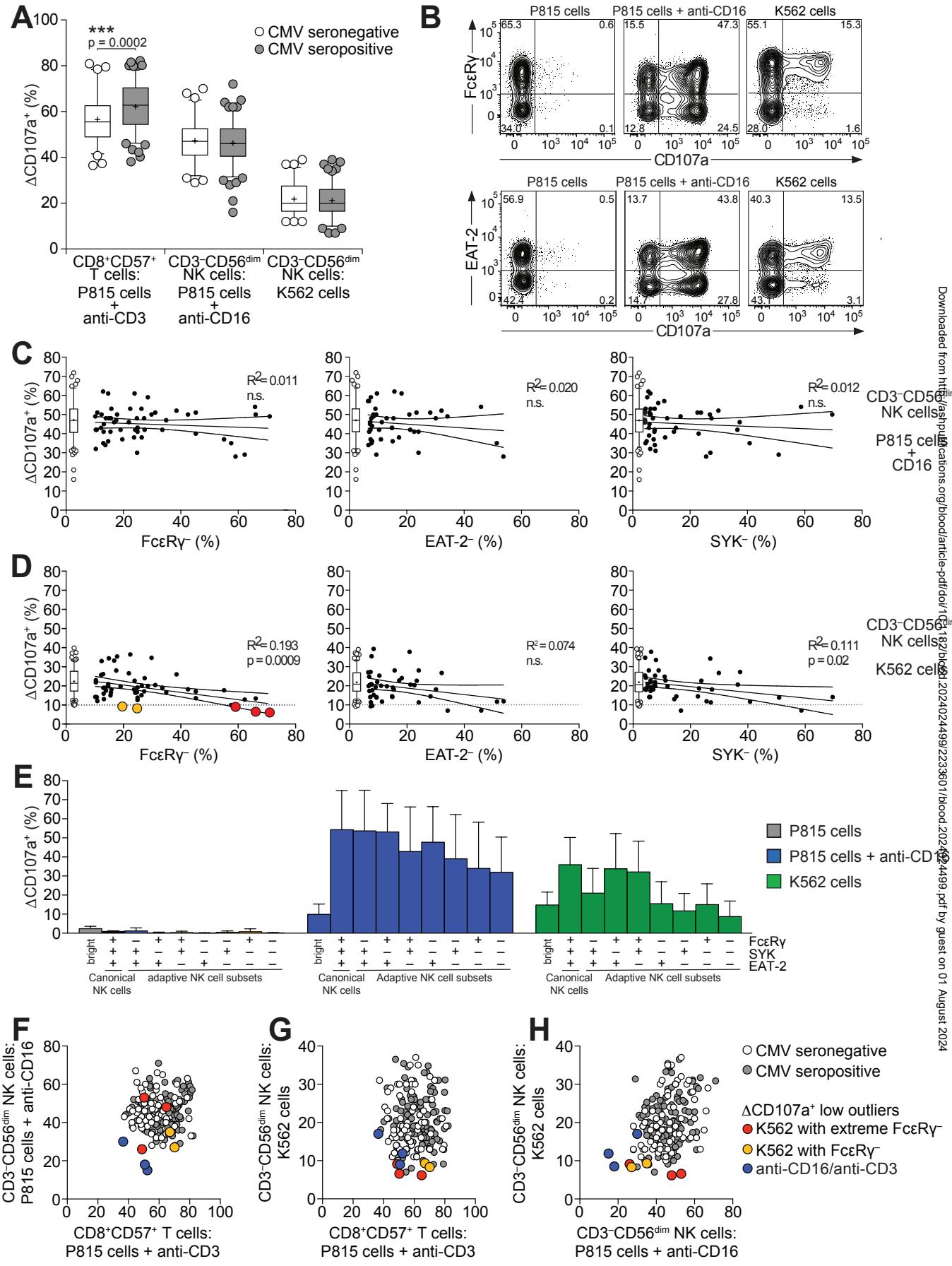


Figure 2. Chiang *et al*.

Figure 3

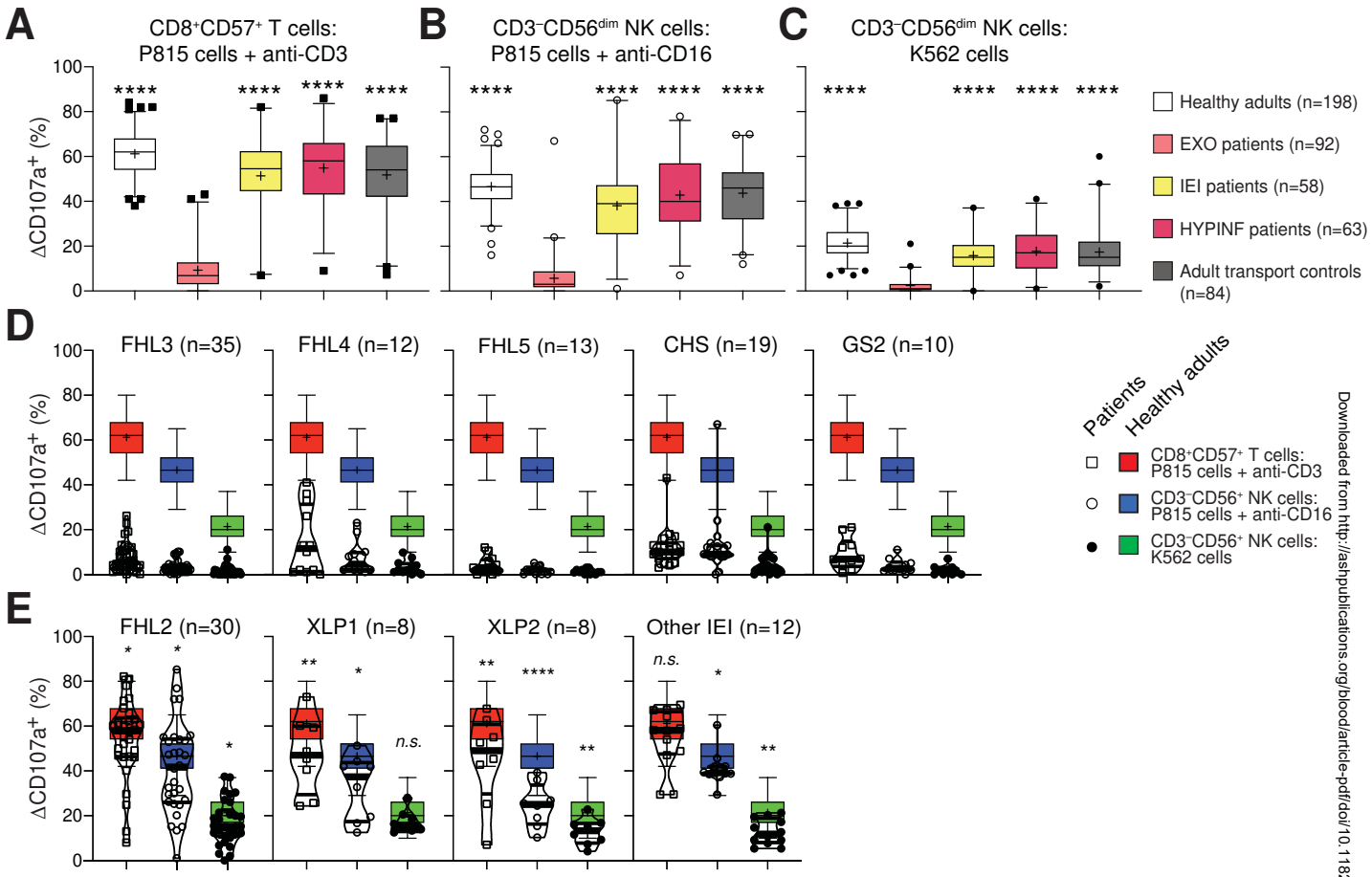


Figure 3. Chiang *et al*.

Figure 4

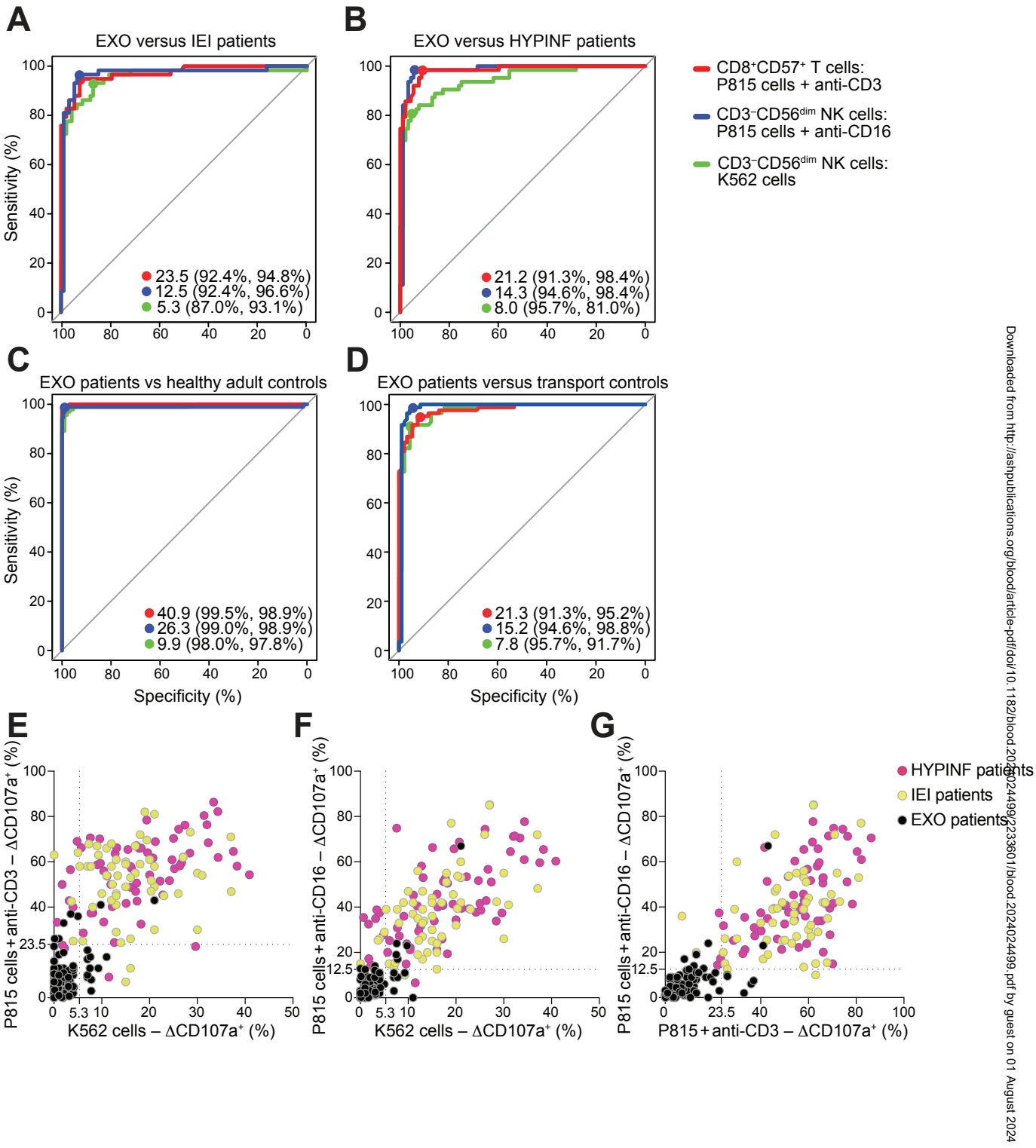


Figure 4. Chiang *et al*.

Figure 5

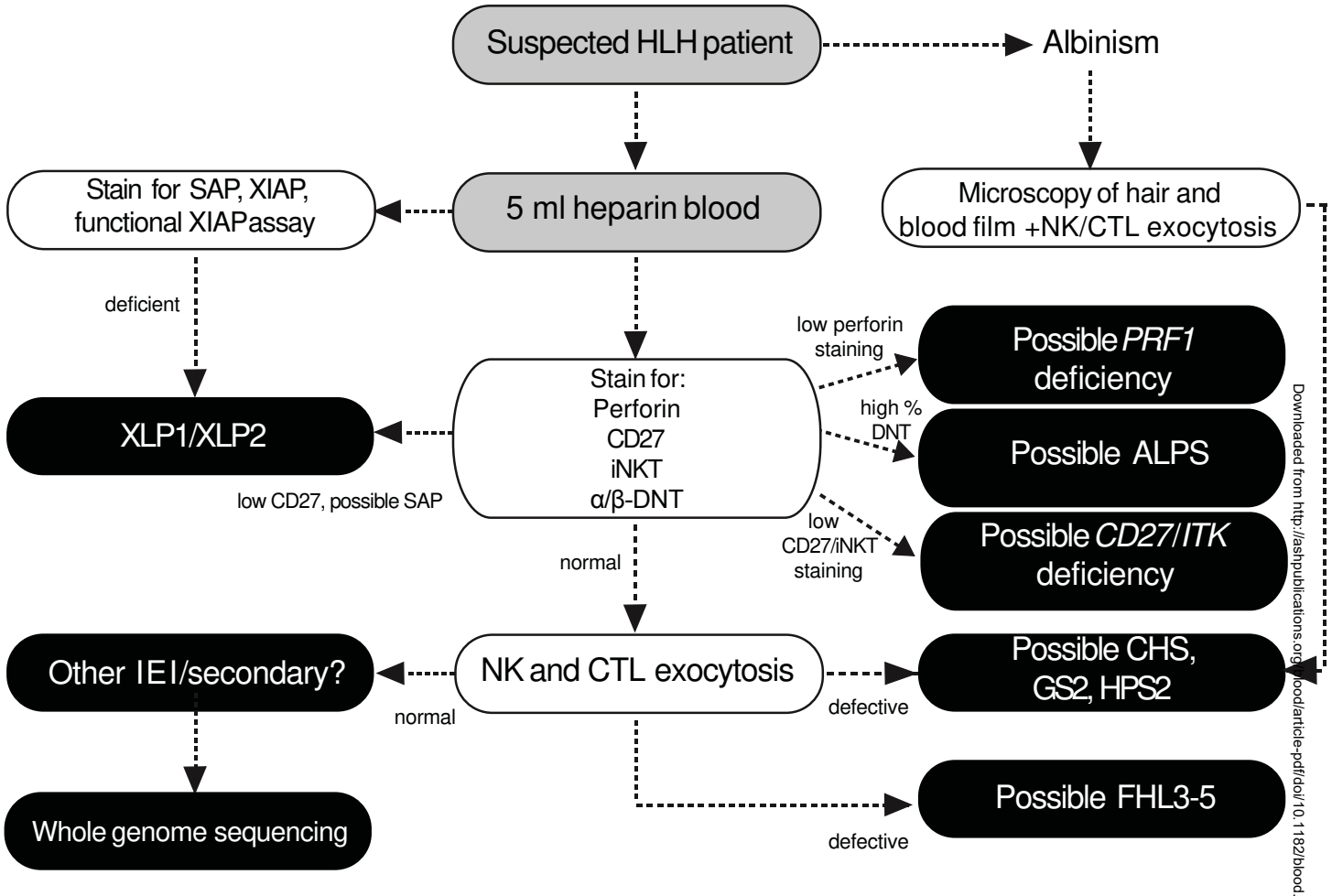


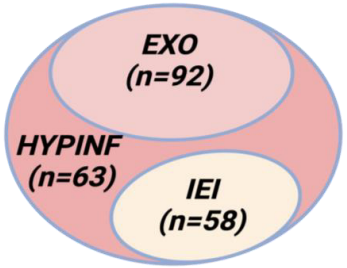
Figure 5. Chiang, *et al*.

Efficacy of T cell assays for the diagnosis of primary defects in cytotoxic lymphocyte exocytosis

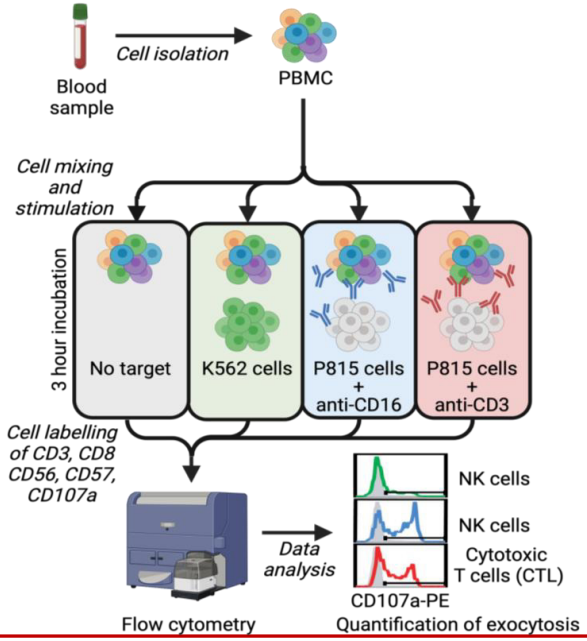
Study subjects

Controls
Healthy blood volunteers
Transport control samples

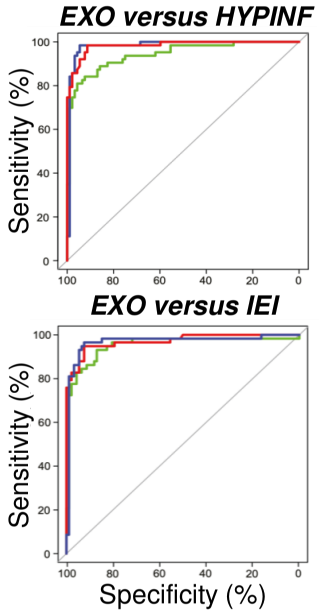
Hyperinflammatory patients
HYPINF – Patients lacking a molecular diagnosis
EXO – Inborn error of immunity (IEI) patients associated with defective exocytosis
IEI – Other IEI patients



Methods



Main Outcomes



Conclusions:

- **Anti-CD16 triggered NK cell and anti-CD3 triggered CTL exocytosis displays the highest accuracy for the diagnosis of primary defects in lymphocyte exocytosis**
- **Combined analyses of CTL and NK cell exocytosis is recommended for the diagnosis of patients with a suspicion of familial HLH**

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