

Chediak-Higashi syndrome: Lysosomal trafficking regulator domains regulate exocytosis of lytic granules but not cytokine secretion by natural killer cells

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Background: Mutations in lysosomal trafficking regulator (*LYST*) cause Chediak-Higashi syndrome (CHS), a rare immunodeficiency with impaired cytotoxic lymphocyte function, mainly that of natural killer (NK) cells. Our understanding of NK cell function deficiency in patients with CHS and how *LYST* regulates lytic granule exocytosis is very limited.

Objective: We sought to delineate cellular defects associated with *LYST* mutations responsible for the impaired NK cell function seen in patients with CHS.

Methods: We analyzed NK cells from patients with CHS with missense mutations in the *LYST* ARM/HEAT (armadillo/huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1) or BEACH (beige and Chediak-Higashi) domains.

Results: NK cells from patients with CHS displayed severely reduced cytotoxicity. Mutations in the ARM/HEAT domain led to a reduced number of perforin-containing granules, which were significantly increased in size but able to polarize to the immunologic synapse; however, they were unable to properly fuse with the plasma membrane. Mutations in the BEACH domain resulted in formation of normal or slightly enlarged granules that had markedly impaired polarization to the IS but could be exocytosed on reaching the immunologic synapse. Perforin-containing granules in NK cells from patients with CHS did not acquire certain lysosomal markers (lysosome-associated membrane protein 1/2) but were positive for markers

of transport vesicles (cation-independent mannose 6-phosphate receptor), late endosomes (Ras-associated binding protein 27a), and, to some extent, early endosomes (early endosome antigen 1), indicating a lack of integrity in the endolysosomal compartments. NK cells from patients with CHS had normal cytokine compartments and cytokine secretion.

Conclusion: *LYST* is involved in regulation of multiple aspects of NK cell lytic activity, ranging from governance of lytic granule size to control of their polarization and exocytosis, as well as regulation of endolysosomal compartment identity. *LYST* functions in the regulated exocytosis but not in the constitutive secretion pathway. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Chediak-Higashi syndrome, lysosomal trafficking regulator, natural killer cell, cytotoxicity, cytotoxic lymphocyte, lysosomes, lytic granules, exocytosis, immune deficiency

Chediak-Higashi syndrome (CHS; OMIM 214500) is a rare lysosomal storage disorder caused by mutations in lysosomal trafficking regulator (*LYST*), which encodes the *LYST* protein.^{1,2} CHS is characterized by oculocutaneous albinism, bleeding diathesis, and immune dysregulation.³⁻⁵ About 85% to 90% of patients have a severe form of the disease (classic CHS), with a fatal hyperinflammatory syndrome termed hemophagocytic lymphohistiocytosis (HLH).^{3,6} Death usually occurs in the first decade of life from infection, bleeding, or development of HLH.^{3,5} The remaining patients with CHS have a milder attenuated form of the disease without HLH (atypical CHS).⁶ A cell biology characteristic of CHS is the presence of giant lysosomes or lysosome-related organelles in several cell types.^{7,8} *LYST* is a 429-kDa protein with several distinct domains implicated in various aspects of vesicular trafficking: ARM/HEAT (armadillo/huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1), PH (pleckstrin homology), BEACH (beige and Chediak-Higashi) and WD-40 (tryptophan-aspartic acid dipeptide repeat),^{2,5,9,10} but its exact function remains to be elucidated.

Natural killer (NK) cells represent a subset of lymphocytes playing a key role in immunosurveillance and host defense against cancer and microbial pathogens.¹¹ Although contributing to the innate immune response, they also modulate the adaptive immune response.¹²⁻¹⁴ NK cells recognize stressed cells through germline-encoded activating and inhibitory cell-surface receptors¹⁵ and use their cytotoxic potential to eliminate abnormal cells and certain activated immune cells. Signals for activation, inhibition, or both are generated at a specialized contact site

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Abbreviations used

ARM/HEAT domain:	Armadillo/huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast kinase TOR1 domain
BEACH domain:	Beige and Chediak-Higashi domain
CHS:	Chediak-Higashi syndrome
CI-MPR:	Cation-independent mannose 6-phosphate receptor
EEA-1:	Early endosome antigen 1
F-actin:	Filamentous actin
HLH:	Hemophagocytic lymphohistiocytosis
IS:	Immunologic synapse
LAMP1/2:	Lysosome-associated membrane protein
LYST:	Lysosomal trafficking regulator
MTOC:	Microtubule organizing center
PH domain:	Pleckstrin homology domain
NK:	Natural killer
Rab:	Ras-associated binding protein
WD-40 domain:	Tryptophan-aspartic acid dipeptide repeat domain

formed between an NK cell and a target cell, which is known as the immunologic synapse (IS).¹⁶ Target cell killing is a multistage process that concludes in exocytosis of perforin- and granzyme-containing lytic granules (secretory lysosomes) at the IS and induction of target cell apoptosis.^{17,18} Defects in lytic granule secretion are associated with often fatal diseases, including familial HLH type 2 to 5, Griscelli syndrome type 2, and CHS.^{18,19} NK cells in patients with CHS have abnormal morphology and function; a study of 2 patients with CHS showed profoundly impaired NK cell cytotoxic activity.^{20,21} In line with the characteristic feature of CHS in other cell types, a single giant granule has been observed in NK cells from patients with CHS,²² and enlarged lytic granules have been reported in cytotoxic T cells.²³ Nevertheless, our understanding of NK cell defects in patients with CHS is limited, and the role of *LYST* in cytotoxic lymphocyte biology is ill defined. Therefore we sought to dissect the effects of *LYST* mutations on different aspects of NK cell function.

METHODS

See the [Methods](#) section in this article's Online Repository at www.jacionline.org for detailed descriptions of methods and reagents.

Subjects and healthy donors

Patients with CHS were enrolled in protocol 00-HG-0153, which was approved by the National Human Genome Research Institute's Institutional Review Board, and provided written informed consent. CHS was considered based on clinical findings and confirmed by means of identification of giant inclusions within leukocytes on peripheral blood smears. Mutations in *LYST* were identified in all subjects and previously reported for some of the cases (see [Table E1](#) in this article's Online Repository at www.jacionline.org).^{6,24,25} Voluntary healthy donors were recruited at the National Institutes of Health, with informed consent provided in accordance with the Declaration of Helsinki.

PBMCs were isolated from whole blood by using the Ficoll-Paque method, and NK cells were isolated by using EasySep Human NK Cell Kits (STEMCELL Technologies, Vancouver, British Columbia, Canada) and cultured in X-VIVO medium (Lonza, Walkersville, Md) supplemented with

IL-2 (100 U/mL). IL-2-cultured NK cells were used in experiments, unless otherwise noted.

Cytotoxicity assays

NK cell cytotoxicity was evaluated by using the DELFIA Assay (PerkinElmer, Waltham, Mass), as previously described.²⁶ Lytic units were calculated as described previously.²⁷ Delivery of granzyme B to target cells was assessed, as previously described.²⁶

Cytokine production and release

For total cytokine levels, 2×10^5 NK cells were first mixed with K562 target cells at a 1:1 ratio for the indicated times at 37°C. The cells were next stained with anti-CD56–allophycocyanin, fixed, permeabilized, and stained with anti-TNF- α –phycoerythrin or anti-IFN- γ –phycoerythrin. Data acquisition and analysis were done with FACSsort (BD Biosciences, San Jose, Calif) and FlowJo software (TreeStar, Ashland, Ore).

Cytokine secretion was evaluated by using the Human TNF- α or IFN- γ ELISA MAX Deluxe kit (BioLegend, San Diego, Calif) after stimulating 0.5×10^6 cells for 20 hours with IL-12 (20 ng/mL), IL-15 (100 ng/mL), and IL-18 (100 ng/mL).

Microscopy and image analysis

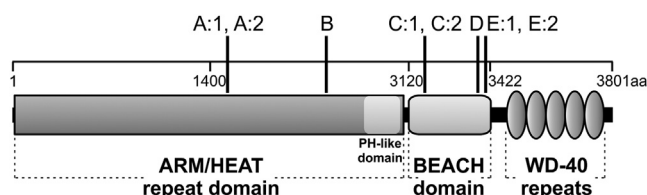
NK cells were left alone or mixed with target cells for 20 minutes at 37°C, followed by adherence to Excell Adhesion Slides (Thermo Scientific, Waltham, Mass) for 10 minutes at 37°C. Cells were fixed, permeabilized, and stained with anti-lysosome-associated membrane protein (LAMP) 1 or anti-LAMP2 antibody, followed by Alexa Fluor 647–conjugated anti-mouse antibody, and anti-pericentrin, followed by Alexa Fluor 568–conjugated anti-rabbit antibody or Alexa Fluor 568–conjugated phalloidin, and then stained with anti-perforin-AF488 antibody. In the experiments determining the location of vesicular compartments, fixed and permeabilized cells were stained with anti-LAMP1 antibody, followed by Alexa Fluor 488–conjugated anti-mouse antibody, and then anti-cation-independent mannose 6-phosphate receptor (CI-MPR), anti-early endosome antigen 1 (EEA-1), or anti-Ras-associated binding protein (Rab) 27a antibody, followed by Alexa Fluor 568–conjugated anti-rabbit antibody, and stained with anti-perforin–Alexa Fluor 647 antibody.

Cells mounted in ProLong Gold (Thermo Scientific) were visualized with a Zeiss LSM710 laser-scanning confocal microscope (Zeiss, Oberkochen, Germany). Images were obtained with a $63 \times$ Zeiss Plan-Apochromat objective and Zeiss Zen software. Perforin polarization and colocalization were assessed, as described previously.^{26,28}

NK cells were labeled for 30 minutes with 200 nmol/L LysoTracker and transferred to polylysine-coated Lab-Tek Chambered Coverglass (Thermo Scientific) in complete X-VIVO medium to assess the size and amount of lytic granules. Cells were imaged in all 3 planes at 37°C by using an Olympus IX81 spinning-disk confocal microscope with $100 \times$ Olympus PlanApo objective (Olympus, Center Valley, Pa). Image acquisition was performed with MetaMorph software by using the streamlining function with the following parameters: 0.2- μ m z-axial dimension, 100-ms exposure per frame, and 25 to 35 frames per image stack. The acquired images were deconvolved with Huygens software (Scientific Volume Imaging b.v., Hilversum, The Netherlands) with distilled experimental point spread function to correct image degradation as point spread function.²⁹ Deconvolved images were analyzed by using Imaris (Bitplane Inc, South Windsor, Conn) and its Spots function.

RESULTS**Subjects' characteristics**

Eight subjects (6 male and 2 female subjects; age range, 21–43 years) from 5 families were studied (see [Table E1](#)). Most patients carried compound heterozygous *LYST* mutations with a



Patient A:1 and A:2: A1454D, Y1687X

Patient B: V2651F

Patient C:1 and C:2: 3276-3277 Δ NAT

Patient D: G3309S, R503X

Patient E:1 and E:2: G3408R, R1104X

FIG 1. *LYST* and position of mutations in patients with CHS examined in the study: schematic representation of the domain organization of *LYST*. The positions of missense mutations identified in individual patients with CHS included in this study (see Table E1) are indicated. The type and location of the mutations in patients with CHS are listed.

truncating mutation combined with a missense mutation, whereas patients from family C carried a homozygous 6-bp *LYST* deletion leading to a predicted loss of 2 amino acids in the BEACH domain of the protein. Patients from families A and B had mutations within the *LYST* ARM/HEAT domain, whereas patients from families C, D, and E had mutations within the *LYST* BEACH domain (Fig 1 and see Table E1). All of these patients presented with atypical CHS, with variable oculocutaneous pigment abnormalities and a history of mild mucosal bleeding. None had recurrent severe or unusual infections, although patient A:1 takes prophylactic antibiotics for recurrent skin infections and patient D had bilateral osteomyelitis in childhood. To date, none of the patients has had HLH. Throughout the text, based on *LYST* mutation positions, patients A:1, A:2, and B are referred to as patients with mutations within the *LYST* ARM/HEAT domain, whereas patients C:1, C:2, D, E:1, and E:2 are referred to as patients with mutations within the *LYST* BEACH domain. One additional subject was a 33-year-old woman with CHS who received a bone marrow transplantation at the age of 10 years; her samples were used as controls in the experiments.

Defective cytotoxicity of NK cells from patients with CHS

We started analysis with an assessment of CD3⁺CD56⁺ NK cell percentages in peripheral blood of patients with CHS. The percentage of NK cells in patients with CHS was decreased compared with that of healthy donors and on or just below the lower end of the normal spectrum (Fig 2, A, left). Nevertheless, NK cells were easily detectable, and the frequency of CD56^{bright} and CD56^{dim} NK cells in patients with CHS was similar to that of healthy donors (Fig 2, A, right).

Next, we tested the cytotoxicity of NK cells isolated from patients with atypical CHS. Compared with healthy donors, patients with CHS had NK cells with significantly decreased natural cytotoxicity (against 721.221 and K562 cells) and almost completely inhibited antibody-dependent cell-mediated cytotoxicity (against SK-OV3 cells; Fig 2, B). Inhibition of lytic activity was prominent for a wide range of effector-to-target ratios (see Fig E1 in this article's Online Repository at www.jacionline.org). The cytotoxicity was restored in NK cells from the patient with CHS who underwent bone marrow transplantation. The decreased cytotoxicity was not due to impaired adhesion with

target cells or modified levels of NK cell receptors because NK cells from patients with CHS conjugated with target cells as normal NK cells (Fig 2, C), and there was no significant difference in cell-surface expression of several activating and inhibitory receptors between NK cells from healthy subjects and patients with CHS (see Fig E2 in this article's Online Repository at www.jacionline.org). Furthermore, silencing of *LYST* in NK cell lines also resulted in severe inhibition of cytotoxicity (see Fig E3 in this article's Online Repository at www.jacionline.org), confirming the importance of the gene for proper NK cell lytic function.

Lytic granules of NK cells from patients with CHS show broad defects related to their morphology and movement

Because inhibition of cytotoxicity in NK cells from patients with CHS was not associated with altered recognition of target cells and a hallmark of CHS is the presence of enlarged lysosome-related organelles, we investigated the morphology and location of lytic granules in NK cells from patients with CHS. We investigated whether the position of *LYST* mutations (either within the ARM/HEAT or BEACH domains of *LYST*) influenced NK cytotoxic granule morphology or dynamics.

Normal NK cells had multiple small granules (352 nm in mean diameter); almost 50% of the granules were smaller than 300 nm, and their size rarely exceeded 600 nm (Fig 3, A), which is similar to former estimates.^{30,31} Most normal NK cells contained 30 to 60 granules (49 ± 17 granules [mean $\pm$ SD]), with several cells containing up to 110 granules (Fig 3, B), which is in line with previous data.³⁰ Normal NK cells accumulated filamentous actin (F-actin; see Fig E4 in this article's Online Repository at www.jacionline.org) and polarized the microtubule organizing center (MTOC) toward the IS in response to target cell stimulation (Fig 4, A). Lytic granules were positive for perforin and granzyme A (see Fig E5 in this article's Online Repository at www.jacionline.org), as well as for the lysosomal markers LAMP1 and LAMP2 (Fig 4, A, and see Fig E4). After target cell recognition, the majority of lytic granules polarized to the IS, forming tight clusters around the MTOC (Fig 4, A, and see Fig E4, B). The average granule distance to the IS was $0.99 \pm 0.7 \mu\text{m}$, which was equivalent to 2.8 diameters of normally sized granules; the distance to the MTOC equaled 3.6 granule diameters ($1.28 \pm 0.7 \mu\text{m}$; Fig 4, B, and see Fig E4, C).

Granule distribution and morphology were different in NK cells from patients with CHS. Unexpectedly, instead of 1 huge granule, as reported previously,²² the size and number of the lytic granules varied between NK cells from patients with CHS with different mutations. NK cells from patients with CHS with *LYST* mutations within the ARM/HEAT domain had markedly enlarged vesicles that were reduced in number. Average granule size was increased to 560 nm, and almost 40% of the granules reached more than 600 nm in diameter (Fig 3, A). The average number of granules was 19 ± 8 (SD), and approximately 50% of the NK cells had fewer than 20 granules, which was not observed in normal NK cells (Fig 3, B). The large granules were positive for perforin and granzyme A and polarized to the IS on NK cell binding to susceptible target cells (Fig 4, A and B, and see Fig E5). The granule distance to the IS was $1.45 \pm 0.9 \mu\text{m}$, which was equivalent to 2.6 diameters of these large granules; the distance to the MTOC equaled 3 granule diameters ($1.72 \pm 0.7 \mu\text{m}$; Fig 4, B, and see Fig E4, C). In contrast, NK

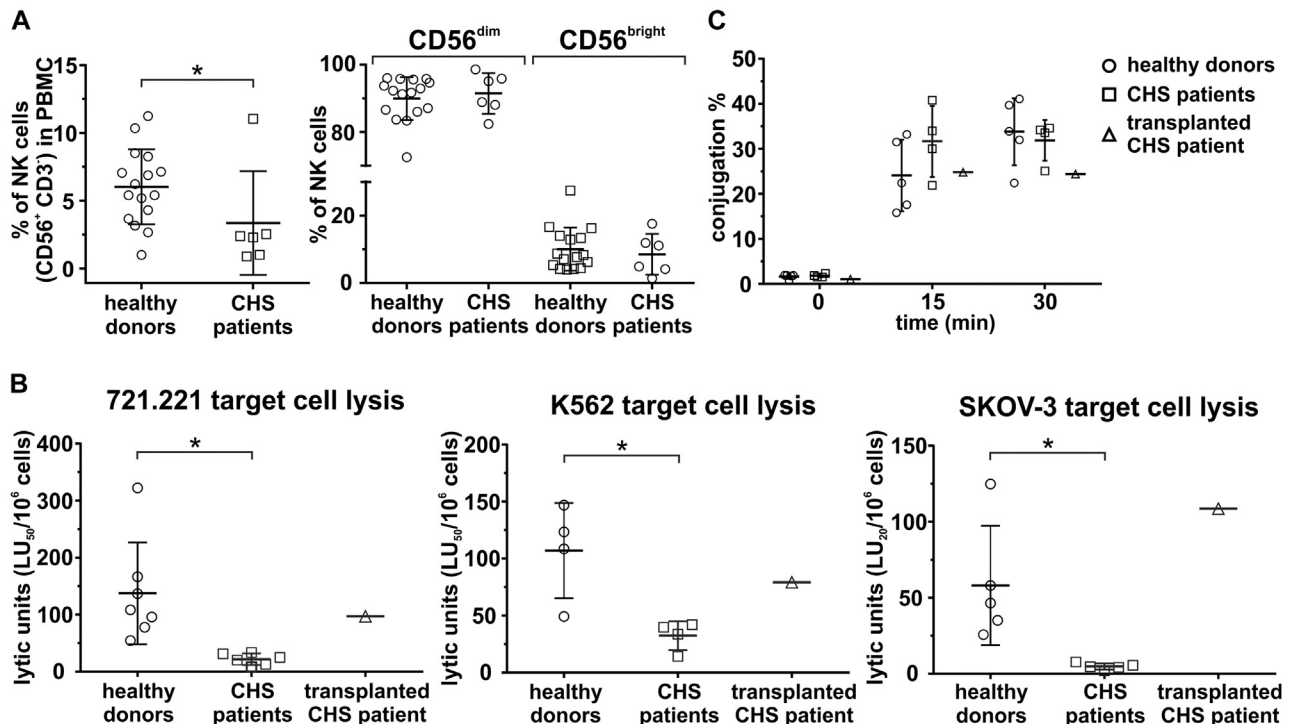


FIG 2. NK cells from patients with CHS have significantly reduced natural and antibody-dependent cell-mediated cytotoxicity. **A**, Percentage of CD3⁺CD56⁺ NK cells in PBMCs and percentage of CD56^{dim} and CD56^{bright} cells in an NK cell population of healthy donors and patients with CHS. **B**, Cytotoxic activity of NK cells against different cell lines. **C**, Percentage of conjugate formation between NK and target cells. Graphs show values for individual patients, with means $\pm$ SDs. * $P < .05$; only significant changes are indicated.

cells from patients with CHS with *LYST* BEACH domain mutations had more numerous granules, most of which appeared to be normal in size or slightly enlarged. The average granule diameter was 397 nm; granule size distribution resembled that of normal NK cells, with increased percentages (approximately 30%) of granules larger than 500 nm (Fig 3, A). The majority of NK cells had between 15 and 30 granules (23 ± 8 granules [mean $\pm$ SD]). Interestingly, despite normal MTOC polarization to the IS in these NK cells, the granules did not translocate properly to the IS because the distance to the IS increased to $2.96 \pm 1.7 \mu\text{m}$, which corresponded to a distance equaling 7.5 average granule diameters. They also remained dispersed throughout the cell and did not converge at the MTOC, as evidenced by an increase in granule distance from the MTOC to 5.7 granule diameters ($2.25 \pm 1.2 \mu\text{m}$; Fig 4, A and B, and see Fig E4, C).

Remarkably, we also observed striking differences in the location of lysosomal markers. Contrary to normal NK cells, NK cells from patients with CHS had granules that were positive for perforin but negative for LAMP1 or LAMP2 and *vice versa* (Fig 4, A, and see Fig E4). NK cells from patients with *LYST* ARM/HEAT, as well as BEACH, domain mutations had significantly decreased colocalization between perforin and LAMP1 or LAMP2 (Fig 4, C). NK cells from patients with CHS accumulated F-actin at the IS (see Fig E4), which, combined with unaffected MTOC polarization to the IS (Fig 4), indicated normal activation of NK cells from patients with CHS. Moreover, as in NK cells from patients with CHS, silencing of *LYST* in NK cell

lines also resulted in defects in acquisition of the lysosomal marker LAMP2 by perforin-positive vesicles but did not compromise the ability of *LYST*-silenced cells to translocate the MTOC or the enlarged granules toward the IS (see Fig E3). The patient with CHS who underwent bone marrow transplantation had NK cells with granules indistinguishable from NK cells isolated from healthy donors, as expected (see Fig E6 in this article's Online Repository at www.jacionline.org).

Perforin-containing granules of NK cells from patients with CHS display mixed vesicular compartment markers

The altered colocalization between perforin and LAMP1/LAMP2 proteins indicated that the granules in NK cells from patients with CHS only partially acquired lysosomal markers. Therefore we asked about the distribution of other vesicular markers in NK cells from patients with CHS. To this end, we investigated the location of CI-MPR (transport vesicle marker), Rab27a (late endosome marker), and EEA-1 (early endosome marker) in NK cells.

In normal unstimulated NK cells, EEA-1-positive endosomes were distinct from lytic granules, and there was a small overlap between perforin-containing granules and CI-MPR- or Rab27a-positive vesicles (Fig 5 and see Fig E7 in this article's Online Repository at www.jacionline.org), which is in agreement with previous studies.^{26,32} In comparison, nearly all perforin-positive granules in NK cells from patients with CHS were positive for

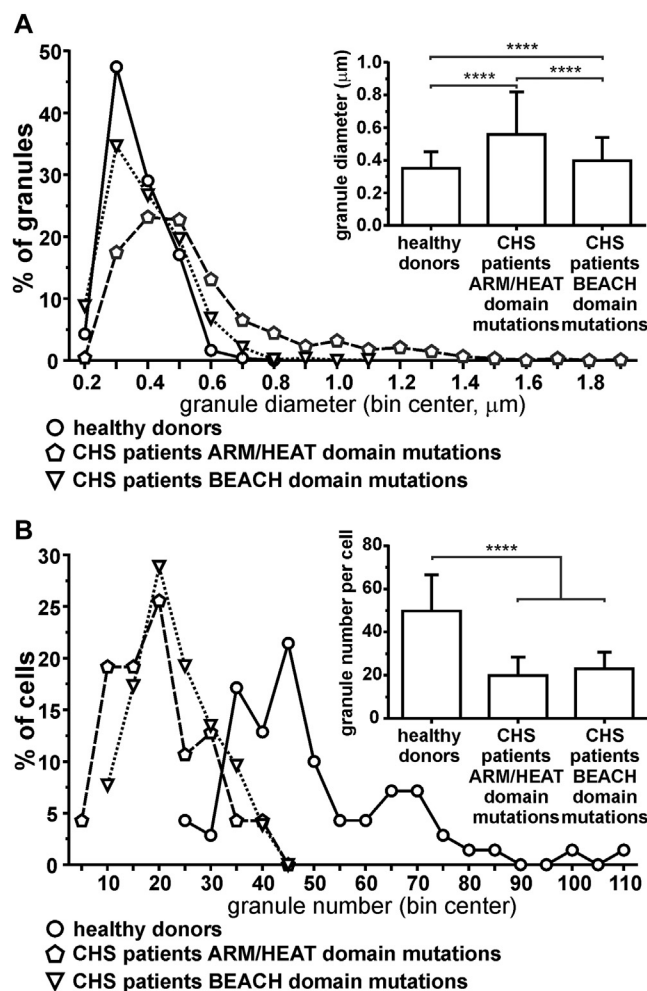


FIG 3. Distinct effects of *LYST* mutations on NK cell lytic granule size and quantity. NK cells from healthy donors or patients with CHS labeled with LysoTracker Red were visualized by using spinning disk confocal microscopy to determine the diameter (**A**) or number (**B**) of lytic granules. Line graphs illustrate the frequency distribution of granule size or amount, inserted bar graphs show mean values for the granule diameter or number, and error bars indicate SDs. **** $P < .0001$.

CI-MPR (Fig 5, A and C, and see Fig E7), and the majority of perforin-positive vesicles were also positive for Rab27a (Fig 5, B and C). Moreover, many of the perforin granules also acquired EEA-1, although at low levels (Fig 5, A and C, and see Fig E7). Thus perforin-containing granules in NK cells from patients with CHS combine features of different endolysosomal compartments, revealing a mixed vesicular identity.

Defects in granule exocytosis and delivery of granzyme B underlies the impaired cytotoxicity of NK cells from patients with CHS

The alterations in lytic granule size or polarization suggested that inhibition of cytotoxicity could be caused by problems with degranulation of NK cells from patients with CHS.³³ The fact that LAMP1 and LAMP2 locations were altered in regard to perforin granules in NK cells from patients with CHS (Fig 4 and see Fig E4) led to our concern that the standard LAMP1 externalization assay might not accurately reflect NK cell degranulation in

patients with CHS. Therefore we used a granzyme B delivery assay, a method that correlates with degranulation and allows for direct visualization of NK cell lytic activity.²⁶

Analysis of granzyme B activity in target cells revealed that only a small portion of NK cells from patients with CHS were able to deliver granzyme to target cells, and there was a notable 2.3-fold decrease in granzyme B delivery (Fig 6, A and B). Degranulation of NK cells from patients with CHS was not totally defective, and some NK cells from patients with CHS were able to release their lytic granules, likely accounting for the observed residual cytotoxicity. Although there was no difference in the percentage of target cells that received granzyme from NK cells with either *LYST* ARM/HEAT or *LYST* BEACH domain mutations, analysis of the fluorescence intensity of cleaved substrate, which translates to the activity of granzyme B in target cells, showed that NK cells of patients with CHS with *LYST* ARM/HEAT domain mutations that had degranulated delivered minimal amounts of granzyme B (Fig 6, C); however, NK cells from patients with CHS with *LYST* BEACH domain mutations that were capable of degranulation delivered granzyme amounts similar to those of normal NK cells (Fig 6, C). There was no decrease in the amount of perforin or granzyme B in NK cells from patients with CHS when compared with that in normal NK cells (data not shown). The delivery of granzyme B from NK cells of a patient with CHS who received a bone marrow transplant was the same as from normal NK cells, indicating that these NK cells had normal cytotoxic potential.

Thus NK cells from patients with CHS with *LYST* ARM/HEAT domain mutations are able to polarize their granules to the IS but cannot fully release them. NK cells from patients with CHS with *LYST* BEACH domain mutations are able to release normal levels of granzyme B but likely do not kill target cells because of defective granule polarization and accumulation at the IS.

Cytokine production and secretion in NK cells from patients with CHS

NK cells are potent producers of several cytokines, including TNF- α and IFN- γ .^{14,34} Because NK cells from patients with CHS showed defective lytic granule polarization or release, we investigated whether similar defects could be observed for cytokine secretion. Recognition of target cells resulted in a significant increase in the percentage of IFN- γ - and TNF- α -positive NK cells from patients with CHS compared with normal NK cells (Fig 7, A, and see Fig E8, A, in this article's Online Repository at www.jacionline.org). The increased percentage of cytokine-producing NK cells from patients with CHS suggested a partial block of exocytosis (caused by enlarged cytokine-positive granules) and/or differences in response to stimulation in NK cells from patients with CHS. NK cells from patients with CHS secreted similar amounts of IFN- γ and TNF- α as normal NK cells in response to cytokine or target cell stimulation (Fig 7, B, and data not shown), indicating that cytokine exocytosis was not overly affected in these cells. There were no differences in cytokine production and secretion among patients with *LYST* ARM/HEAT or BEACH domain mutations. Furthermore, IFN- γ -containing vesicles were similar in NK cells from patients with CHS and normal NK cells, and there was no fusion between perforin and IFN- γ granules (Fig 7, C). Thus the increased amount of cytokine-producing NK cells in patients with CHS was not due to mixed compartmentalization or retention of

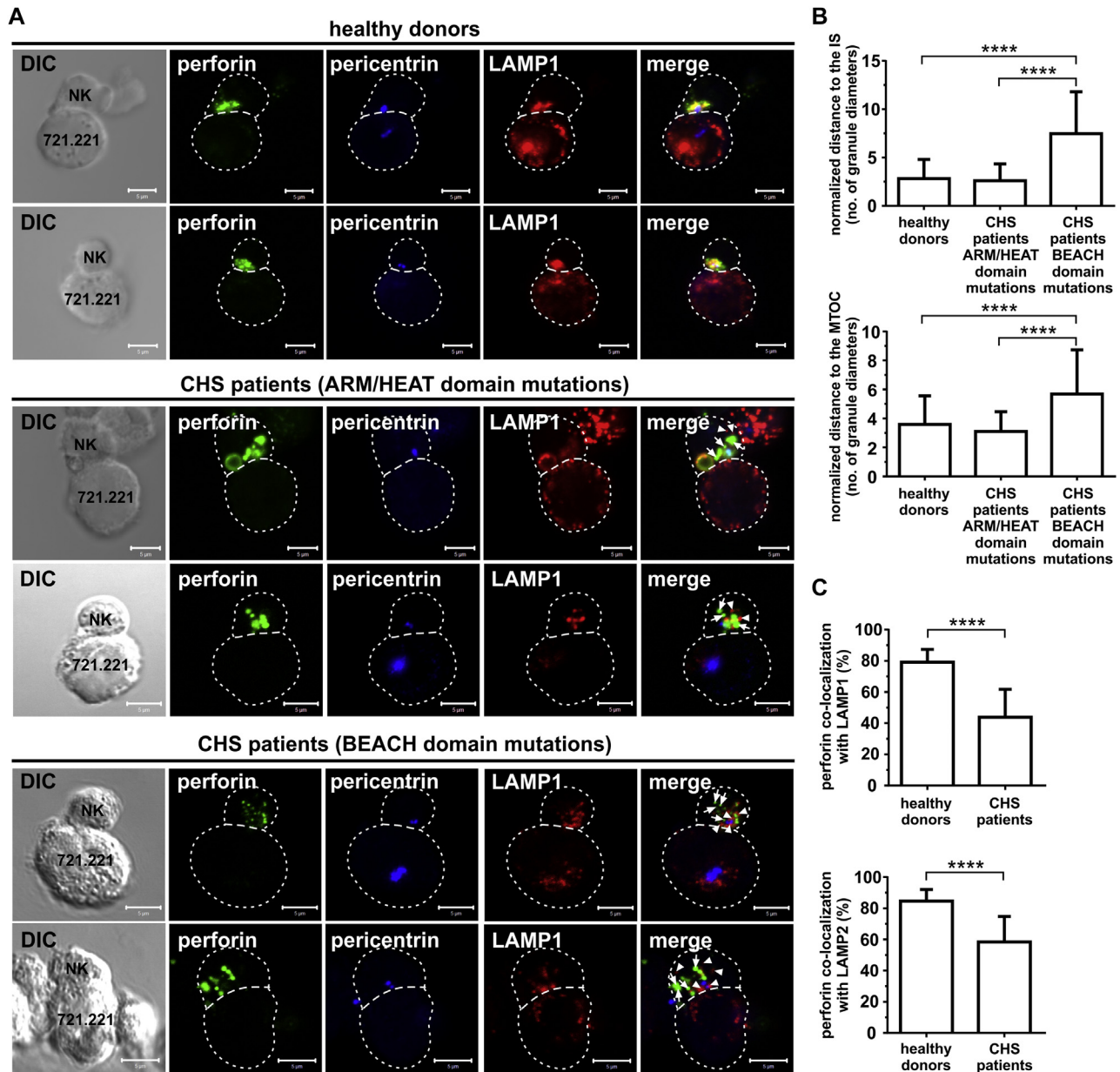
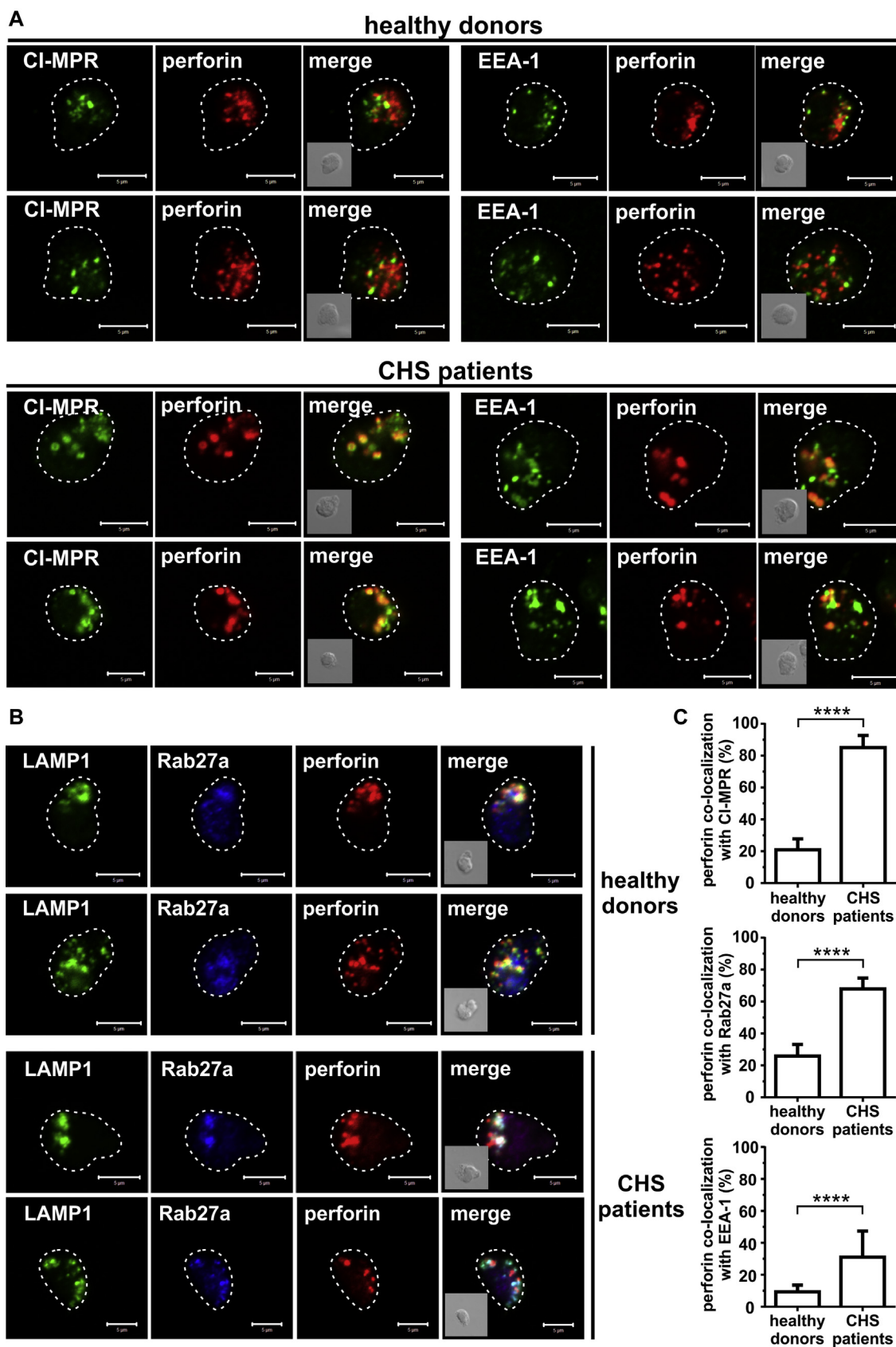


FIG 4. NK cells from patients with CHS display a heterogeneous spectrum of defects related to lytic granule size, polarization, and acquisition of lysosomal markers. **A**, NK cells isolated from healthy donors or the indicated patients with CHS mixed with target cells were stained with antibodies against perforin (green), pericentrin (MTOC marker; blue), and LAMP1 (red). Short-dashed lines indicate cell outlines, and long-dashed lines show the position of the IS. Arrows and arrowheads indicate vesicles positive for perforin and negative for LAMP1 or vice versa, respectively. Scale bars = 5 μ m. Two representative examples for each group are shown. **B**, Perforin coalescence around the MTOC and polarization to the IS after interaction between NK and target cells, as shown in Fig 4, A, and Fig E4. Graphs illustrate mean values, and error bars show SDs. **** $P < .0001$. **C**, Percentage of colocalization between perforin and LAMP1 or LAMP2. Data are shown as means + SDs. **** $P < .0001$.

cytokine vesicles. However, we found that NK cells from patients with CHS responded faster to target cell stimulation than normal NK cells. There was no difference in cytokine production between NK cells from patients with CHS and normal NK cells immediately after mixing with target cells; nevertheless, within 2 to 3 hours of stimulation, the percentage of cytokine-producing NK cells from patients with CHS increased significantly compared with that in normal NK cells, and the amount

of TNF- α - and IFN- γ -positive NK cells from patients with CHS peaked at 4 and 6 hours of stimulation, respectively. Within 12 to 18 hours, however, normal NK cells reach similar percentages of cytokine-producing cells (Fig 7, D), which could explain the lack of an overt difference in the amount of cytokine secreted by normal cells and NK cells from patients with CHS 20 hours after stimulation (Fig 7, B). Of note, NK cells from patients with CHS did not spontaneously secrete TNF- α or IFN- γ (data



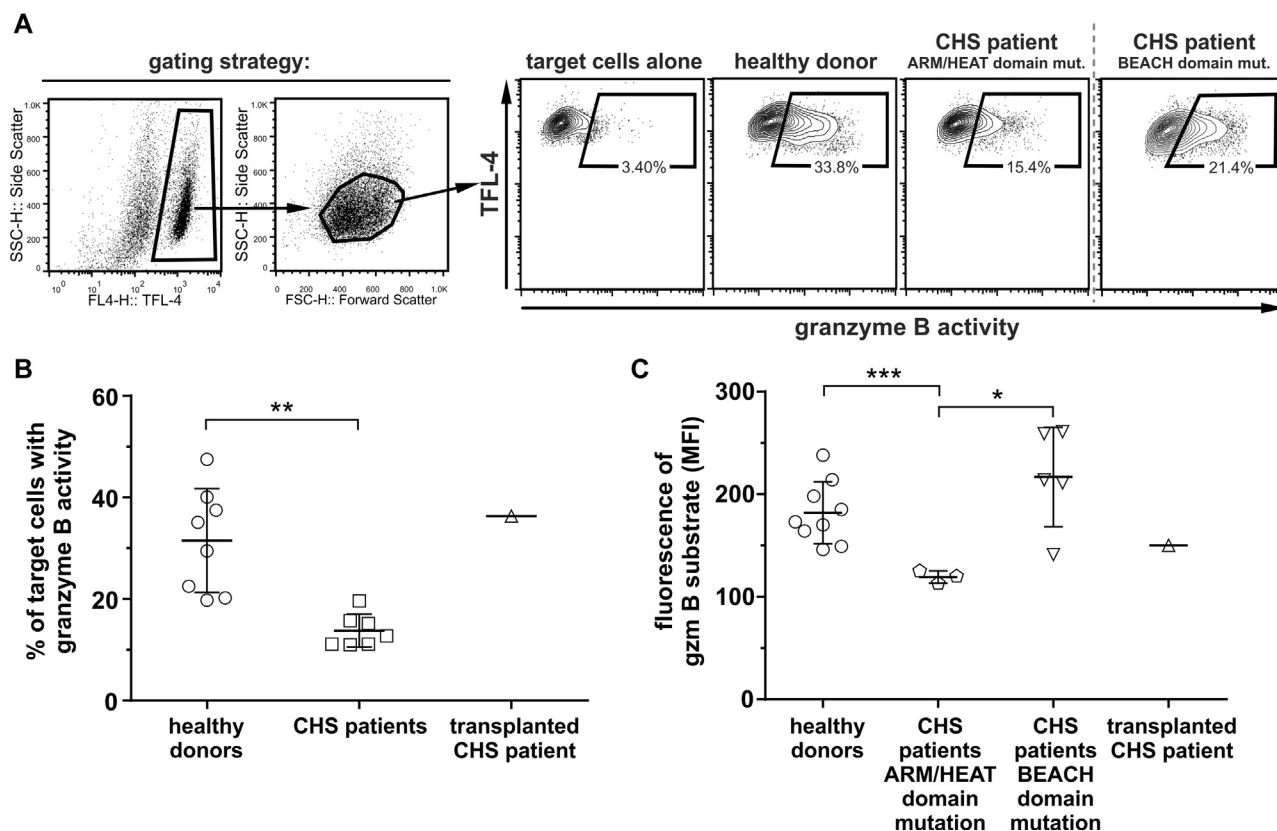


FIG 6. Impaired delivery of granzyme B from NK cells from patients with CHS to target cells. **A**, Granzyme B delivery from normal NK cells or NK cells from patients with CHS to target cells. Plots show the gating strategy and illustrate representative results. *FSC*, Forward scatter; *SSC*, side scatter; *TFL-4*, target fluorescent label/light 4. **B**, Quantification of data shown in Fig 6, **A**. There were no differences between patients with *LYST* ARM/HEAT or BEACH domain mutations. **C**, Target cells positive for granzyme B activity, as determined in Fig 6, **A**, were analyzed for the mean fluorescence intensity (MFI) of granzyme B substrate. Graphs in Fig 6, **B** and **C**, show values for individual persons, with means $\pm$ SDs. * $P < .05$, ** $P < .01$, and *** $P < .001$.

not shown), and TNF- α and IFN- γ levels in sera of patients with CHS were minimal and the same as levels in sera of healthy donors (see Fig E8, **B**). In summary, although *LYST* is important for controlling the size and release of lytic granules in NK cells, cytokine-containing granules and their secretion are not adversely affected by the loss of *LYST* function.

DISCUSSION

CHS, which is caused by mutations in the *LYST* gene, is characterized by formation of giant lysosomes and defects in lysosomal cargo trafficking and delivery. Disruption of vesicular traffic in patients with CHS influences both innate and adaptive immunity, leading to primary immunodeficiency. Surprisingly,

little is known about the functions of *LYST* in human lymphocytes.

Impaired cytotoxicity of NK (Fig 2 and see Fig E1) or T cells in patients with CHS has been reported before,^{20-22,35-37} but the majority of reports have described a single case with 1 or 2 classically affected subjects.^{20,21,35,36} Our study on a group of patients with atypical CHS provides several important insights into mechanisms underlying defective cytotoxicity of NK cells in patients with CHS. *LYST* mutations result in heterogeneous cellular phenotypes of NK cells, with a spectrum of defects related to granule morphology, transport, and exocytosis but not NK cell activation or recognition of target cells (Fig 8). *LYST* is a large protein with several distinct domains (ie, ARM/HEAT, PH, BEACH, and WD-40; Fig 1),^{2,5,9,10} but the function of individual *LYST* domains has

FIG 5. Perforin-containing granules of NK cells from patients with CHS are positive for markers of several different vesicular compartments. NK cells were stained with antibodies against perforin (red) and different vesicular compartments: **A**, Cl-MPR (transport vesicle marker; green; left panels) or EEA-1 (early endosome marker; green; right panels); **B**, LAMP1 (late endosome/lysosome marker; green) and Rab27a (late endosome/lysosome-related organelle marker; blue). Short-dashed lines indicate cell outlines, and inserts show DIC images. Scale bars = 5 μ m. Two representative examples for each group are shown. There were no noticeable differences between patients with *LYST* ARM/HEAT or BEACH domain mutations. **C**, Percentage of colocalization between perforin and Cl-MPR, Rab27a, or EEA-1. Data are shown as means $\pm$ SDs. **** $P < .0001$.

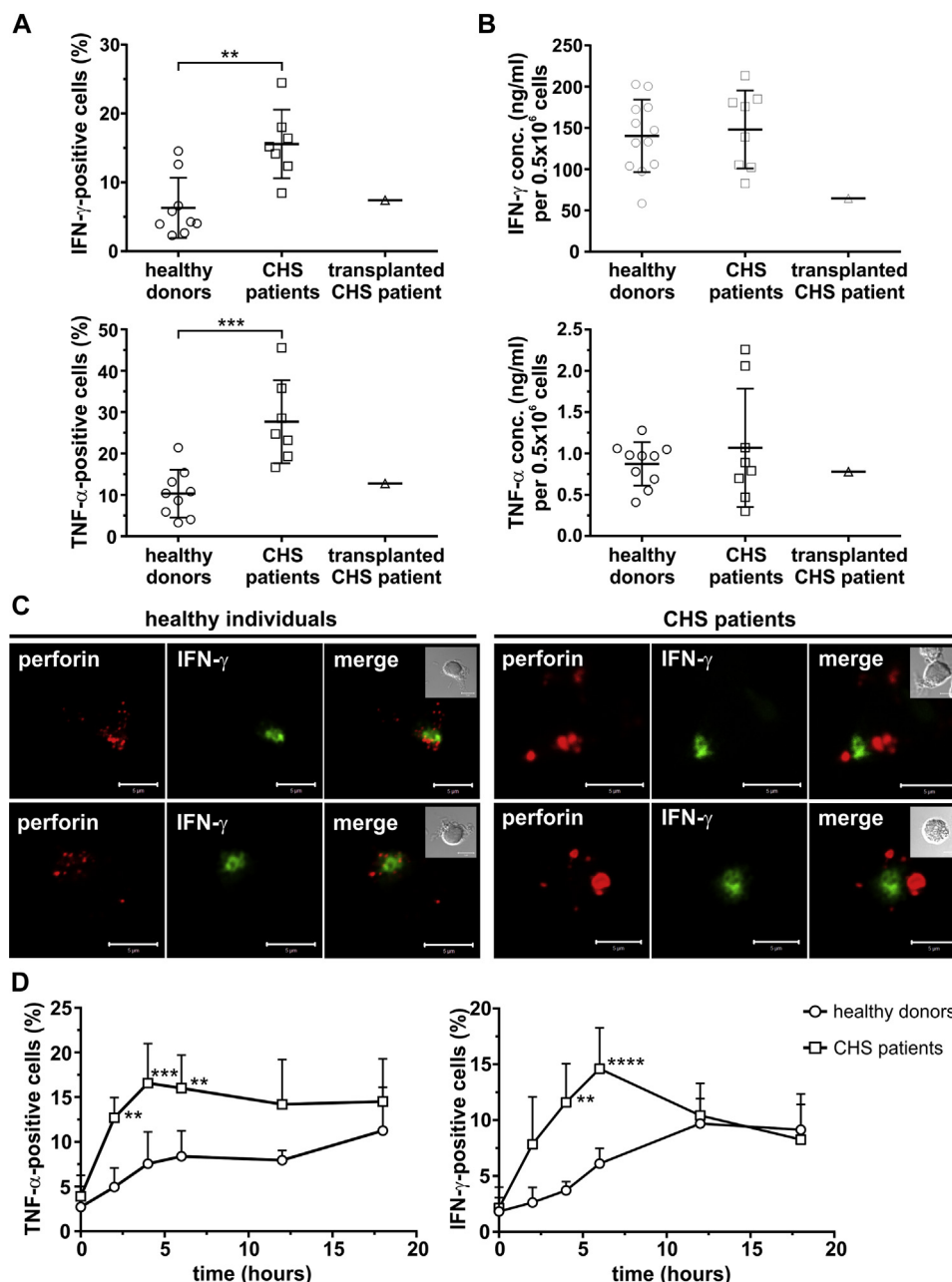


FIG 7. NK cells from patients with CHS produce cytokines in response to target cell stimulation faster than normal cells and do not show evidence of a block in cytokine secretion or abnormal IFN- γ -positive compartments. **A**, IFN- γ and TNF- α production by NK cells cocultured with target cells. ** $P < .01$ and *** $P < .001$. **B**, IFN- γ and TNF- α secretion by NK cells stimulated with IL-12 and IL-18. Graphs in Fig 7, A and B, show values for individual persons, with means $\pm$ SDs. **C**, NK cells stimulated with IL-12 and IL-18 were stained with antibodies against perforin (red) and IFN- γ (green). Scale bars = 5 μ m; inserts show DIC images. **D**, Time course of IFN- γ and TNF- α production by NK cells. Error bars represent SDs. ** $P < .01$, *** $P < .001$, and **** $P < .0001$; only significant values are indicated.

not been elucidated. Uniquely, our data indicate that a correlation exists among granule size, number, and location of *LYST* mutations. Missense mutations within the *LYST* BEACH domain lead to the formation of lytic granules with relatively normal size and number, whereas ARM/HEAT domain missense mutations result in granules of markedly increased size and decreased number (Fig 3). Previously, 1 giant lytic granule had been detected in NK cells from patients with CHS²²; however, the

position of the *LYST* mutations was not determined in those cases. The fact that many of those patients with CHS were children suggest most likely that they had the classic form of CHS. In addition, there appears to be a genotype-phenotype correlation between *LYST* mutations and clinical manifestations of CHS,^{6,38} and thus cellular phenotypes of patients with classic and atypical CHS could be quite different. Furthermore, the large granules in NK cells from patients with CHS with *LYST* ARM/HEAT domain

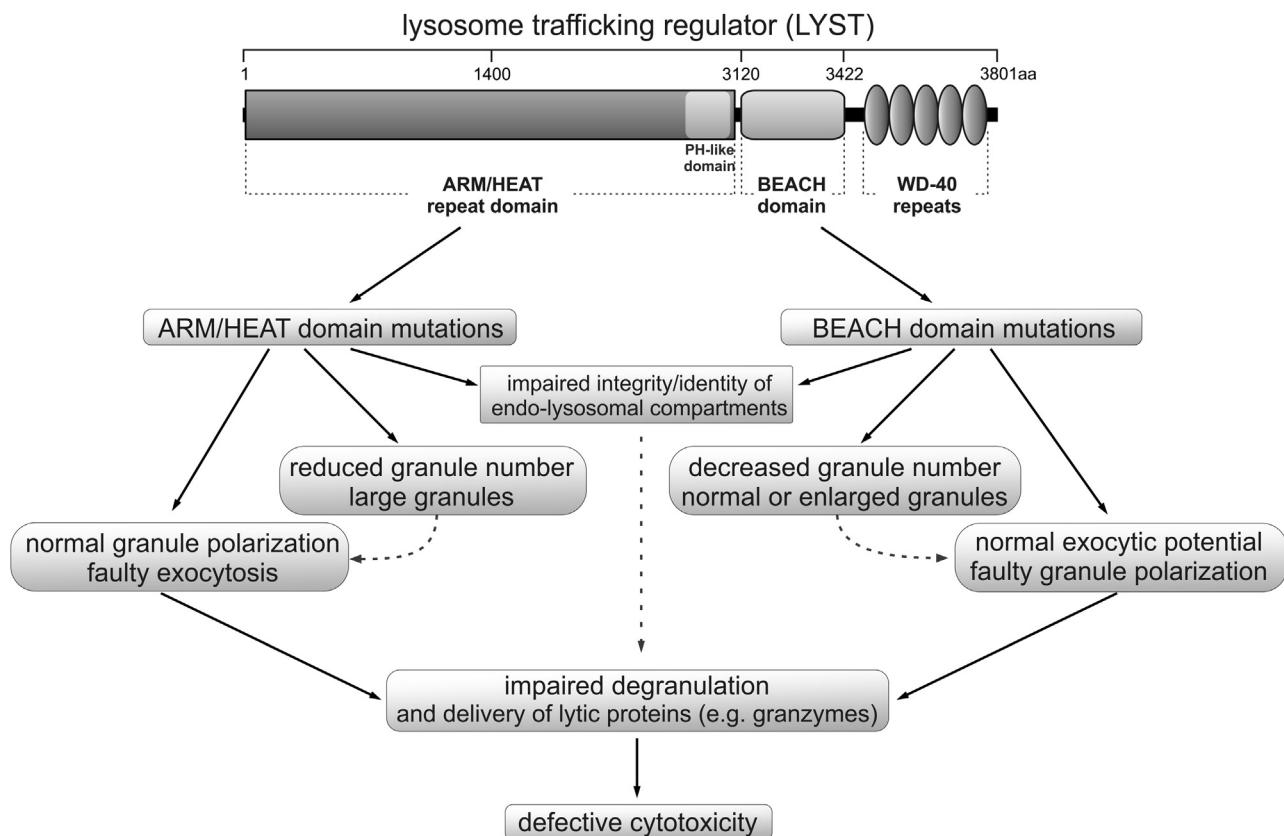


FIG 8. Association of mutation positions with the multiple roles *LYST* plays in regulated exocytosis of NK cells. The diagram illustrates a schematic summary of the findings in patients with atypical CHS. Mutations within the *LYST* ARM/HEAT domain lead to generation of large granules with severely reduced granule numbers. Giant granules are able to polarize to the cell-cell contact area yet are unable to undergo exocytosis; thus faulty granule release underlies the defective cytotoxicity of NK cells from patients with CHS with *LYST* ARM/HEAT domain mutations. On the other hand, mutations within the *LYST* BEACH domain result in generation of decreased amounts of granules with normal or slightly enlarged size. Although those granules are exocytosed normally, they show faulty polarization to the cell-cell contact site and remain dispersed in the cell. Thus in the case of patients with CHS with *LYST* BEACH domain mutations, defective cytotoxicity results from the inability of NK cells to properly translocate lytic granules to the contact site with target cells. Therefore although mutations in different domains of *LYST* produce different cellular phenotypes, they all result in common outcomes, such as compromised integrity of the endolysosomal compartments and impaired granule exocytosis, ultimately leading to defective delivery of lytic proteins to the target cells and impaired cytotoxicity.

mutations are capable of translocation to the IS, whereas the normally sized or slightly enlarged granules resulting from *LYST* BEACH domain mutations show severely impaired polarization to the MTOC and IS and remain dispersed in the cell (Fig 4 and see Fig E4). These results demonstrate that *LYST* is involved in orchestrating granule movement in addition to its role in controlling the size of the lysosome-related organelles (Fig 8).

It remains to be determined whether *LYST* is able to mediate those functions directly or indirectly through interactions with other proteins. *LYST* has been recently postulated to act as a scaffold protein,¹⁰ which could explain the pleiotropic effect caused by *LYST* mutations. For instance, the ARM/HEAT and PH domains have been shown to be involved in vesicular trafficking and membrane lipid binding, as well as protein-protein interactions, respectively.³⁹⁻⁴² Mutations within this region could affect the function of these 2 domains directly and/or *LYST* conformation and domain positioning, resulting in absent or improper interactions between *LYST* and the protein

or proteins required for vesicular fusion or fission. PH domains are known to bind a variety of small GTPases,⁴³ which in turn could regulate vesicular trafficking and fusion.^{44,45} In this regard an overexpression of active Rab14 in *Dictyostelium* species resulted in formation of giant lysosomes, which is reminiscent of those caused by mutation in the *LYST* ortholog *LvsB*, whereas overexpression of a dominant negative form of Rab14 rescued the *LvsB* phenotype, restoring the size and morphology of postlysosomal compartments.⁴⁶ Whether the same occurs in human cells remains to be determined, but the existence of a functional link among *LYST*, Rab GTPases, and vesicle size is a very interesting notion that warrants further examination.

Little is known about the role of *LYST* C-terminal segments. BEACH domain function is completely unknown. Our findings suggest that it might be less important for mediating vesicular fusion but rather play an upstream role in granule movement (Figs 3-5). Another explanation would be that mutations in the BEACH domain change *LYST* conformation, resulting in

mispositioning of the WD-40 domain, which plays a role in multi-protein interactions and vesicle trafficking.⁴⁷ Thus the C-terminal part of *LYST* could be required for binding to proteins involved in vesicular transport, such as motor proteins essential for lytic granule movement to the MTOC and IS, such as dynein.²⁸

Alternatively, altered *LYST* protein levels could be responsible for the observed effects between patient groups. For example, patients with *LYST* ARM/HEAT domain mutations could have less *LYST* than patients with *LYST* BEACH domain mutations, which could explain the more profound cellular phenotype observed in those patients. Unfortunately, a reliable anti-*LYST* antibody is not available, and therefore information about *LYST* levels in NK cells from patients with CHS is currently not obtainable. Nevertheless, altered protein stability, resulting from conformational changes, misfolding, or both, is a consequence of gene mutations. Thus the conclusion about the relationship between the genotype and phenotype in NK cells from patients with CHS remains valid and is supported by a consistent cellular phenotype observed in patients with CHS with *LYST* ARM/HEAT or BEACH domain mutations.

We observed that only a portion of NK cells from patients with CHS were able to release their lytic granules and deliver granzyme B to target cells, indicating that defective lytic granule exocytosis (Fig 6 and Bryceson et al³³) underlies the impaired cytotoxic function of NK cells. Importantly, our data demonstrate that several issues could be responsible for this defect, including the large granule size and concomitant decrease in granule numbers observed in NK cells from patients with CHS with *LYST* ARM/HEAT domain mutations. Although the large granules are able to translocate to the IS, they are not able to be efficiently released. The reason for the defective exocytosis in this case is not clear. One speculation would be a mechanical hindrance caused by increased granule size. The NK cell IS has been shown to contain a meshwork of F-actin through which granules have to pass to be released.^{30,31} The number of actin openings at the IS passable for granules decreases drastically with increased granule diameter,³¹ and thus even if the large granules are able to translocate to the IS, the limited area of actin clearance likely blocks the passage of such big vesicles and restricts their release. Indeed, NK cells from patients with CHS with large granules release markedly less granzyme than normal NK cells (Fig 6), which is reminiscent of a partial fusion pore opening,⁴⁸ and could support the above hypothesis. Interestingly, NK cells from patients with CHS with normal or slightly increased granule size deliver normal amounts of granzyme B (Fig 6), suggesting that their lytic granules are able to penetrate the actin meshwork for complete release. In this case, however, the decreased exocytosis is due to faulty coalescence of granules around the MTOC and translocation to the IS.

This defective exocytosis could also be due to mislocalization of proteins required for the fusion of lytic granules with the plasma membrane.¹⁸ For instance, Rab27a is essential for granule exocytosis in cytotoxic lymphocytes.^{19,45,49} Although the majority of large perforin-positive granules are also positive for Rab27a in NK cells from patients with CHS, the smaller perforin-containing vesicles are devoid of Rab27a (Fig 5), which could affect their release even though their size is likely suitable to pass through the F-actin network. In support of this hypothesis, a very recent report has shown that overexpression of Rab27a and other proteins critical for granule exocytosis helps to restore the degranulation potential of T cells from patients with CHS.⁵⁰

A very important finding is that the lytic granules in NK cells from patients with CHS display markers of usually distinct vesicular compartments.

The nature of enlarged vesicles in patients with CHS has been a matter of active debate, and 2 mechanisms have been proposed to be responsible for their formation. According to the fusion model, the large vesicles are derived from uncontrolled fusion of lysosomes caused by deficient *LYST* function.^{46,50-53} The fission model maintains that *LYST* plays a role in vesicular fission and that there is an incorrect separation of lysosomal membranes after normal fusion events without *LYST*.^{23,54,55} Our data show that in NK cells from patients with CHS, perforin-positive granules amass markers of several vesicular compartments, including proteins from late endosomes, transport vesicles, and even early endosomes (Fig 5 and see Fig E7). The acquisition of markers of trans-Golgi-derived transport vesicles and late endosomal proteins, as well as the presence of low levels of the early endosomal marker EEA-1, suggests that the granules are formed through aberrant fusion of several vesicular compartments. Indeed, impaired integrity, maturation, or both of endolysosomal compartments appears to be common features of cytotoxic lymphocytes from patients with CHS because enlarged vesicles in cytotoxic T cells from patients with CHS also acquire markers of late endosomes, recycling endosomes, and lysosomes.⁵⁰ Interestingly, the function of those mixed-identity vesicles appears to be relatively unaffected. LysoTracker, an acidotropic probe, stained both normal NK cells and NK cells from patients with CHS to the same extent (data not shown), indicating that pH of the granules in NK cells from patients with CHS is not affected, which agrees with reports showing that the acidification of large granules in cells from patients with CHS is not changed.^{56,57} We did not observe any difference in granzyme B activity between normal NK cells and NK cells from patients with CHS, and others have shown that processing and enzymatic activity of several lysosomal enzymes is not affected in cells from patients with CHS,^{23,57} implying that despite their mixed identity and enlarged size, the granules are functional in patients with CHS.

In contrast to abnormalities in secretory granule dimensions and exocytosis, cytokine secretion and cytokine-positive compartments do not appear to be overly affected in NK cells from patients with CHS (Fig 7). In NK cells, as well as several types of innate immune cells, cytokines are sorted away from the contents of lytic granules and traffic through a constitutive secretion pathway involving transfer through recycling endosomes to the plasma membrane.^{58,59} Our data demonstrate that, in contrast to the regulated exocytosis pathway, *LYST* is not required for the constitutive exocytosis pathway, which agrees with the ability of patients with CHS to have IFN- γ -driven hyperinflammatory syndromes. Interestingly, we did not detect altered levels of TNF- α or IFN- γ in the sera of patients with CHS examined in this study, suggesting that immune cells in patients with CHS do not spontaneously secrete these cytokines and likely require a trigger (eg, a viral infection) to elicit their response and induce cytokine secretion, which, when uncontrolled, could lead to hyperinflammation.¹⁷

CHS is undoubtedly a complex disease with many unanswered questions. For instance, is there a particular reason why patients with atypical CHS do not have HLH? One conjecture would be that CHS cytotoxic lymphocytes retain some degree of functionality and that their partial lytic activity could prevent

excessive accumulation of overactivated histiocytes. Is the limited activity of cytotoxic lymphocytes adequate to protect against HLH or only to delay its onset?³⁷ In the majority of cases (classic CHS), HLH develops in infancy, but it is believed that HLH could develop at any age in patients with CHS. In such a case what is the risk for patients with atypical CHS to have HLH? Moreover, it is not clear to what extent *LYST* deficiency affects other cells of the immune system (eg, dendritic cells and macrophages), production of proinflammatory or anti-inflammatory cytokines, and how those events affect the susceptibility to or protection from HLH. Further studies are required to address these important questions.

In summary, through studying patients with unique atypical CHS with missense mutations affecting specific domains of the *LYST* protein, we have gained novel insight into a cellular phenotype of NK cells from patients with CHS. Our data show that NK cells from patients with CHS display a very complex and heterogeneous range of defects related to lytic granule polarization, size, and acquisition of endolysosomal markers, which likely provide a cumulative effect resulting in severely impaired NK cell cytotoxicity (Fig 8).

We thank the patients with CHS and their families for the blood sample donations and their support and the National Institutes of Health Clinical Center, Department of Transfusion Medicine, for providing the healthy donor blood samples.

Key messages

- *LYST* is involved in the regulation of cytotoxic granule size, movement, and exocytosis, as well as maintenance of endolysosomal compartment integrity, but does not control the size of cytokine compartments.
- *LYST* mutations affect multiple aspects of NK cell cytotoxic activity but not cytokine secretion.

REFERENCES

- Barbosa MD, Nguyen QA, Tchernev VT, Ashley JA, Detter JC, Blaydes SM, et al. Identification of the homologous beige and Chediak-Higashi syndrome genes. *Nature* 1996;382:262-5.
- Nagle DL, Karim MA, Woolf EA, Holmgren L, Bork P, Misumi DJ, et al. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nat Genet* 1996;14:307-11.
- Introne WJ, Westbrook W, Golas GA, Adams D. Chediak-Higashi syndrome. In: Pagon RA, Bird TD, Dolan CR, Stephens K, Adam MP, editors. *GeneReviews*. Seattle: University of Washington; 1993.
- Huizing M, Helip-Wooley A, Westbrook W, Gunay-Aygun M, Gahl WA. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. *Annu Rev Genomics Hum Genet* 2008;9:359-86.
- Kaplan J, De Domenico I, Ward DM. Chediak-Higashi syndrome. *Curr Opin Hematol* 2008;15:22-9.
- Karim MA, Suzuki K, Fukai K, Oh J, Nagle DL, Moore KJ, et al. Apparent genotype-phenotype correlation in childhood, adolescent, and adult Chediak-Higashi syndrome. *Am J Med Genet* 2002;108:16-22.
- Windhorst DB, Zelikson AS, Good RA. Chediak-Higashi syndrome: hereditary gigantism of lysoplasmic organelles. *Science* 1966;151:81-3.
- Burkhardt JK, Wiebel FA, Hester S, Argon Y. The giant organelles in beige and Chediak-Higashi fibroblasts are derived from late endosomes and mature lysosomes. *J Exp Med* 1993;178:1845-56.
- Ward DM, Griffiths GM, Stinchcombe JC, Kaplan J. Analysis of the lysosomal storage disease Chediak-Higashi syndrome. *Traffic* 2000;1:816-22.
- Cullinane AR, Schaffer AA, Huizing M. The BEACH is hot: a *LYST* of emerging roles for BEACH-domain containing proteins in human disease. *Traffic* 2013;14:749-66.
- Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* 2008;9:503-10.
- Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol* 2002;71:173-83.
- Cooper MA, Fehniger TA, Fuchs A, Colonna M, Caligiuri MA. NK cell and DC interactions. *Trends Immunol* 2004;25:47-52.
- Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* 2010;115:2167-76.
- Lanier LL. NK cell recognition. *Annu Rev Immunol* 2005;23:225-74.
- Krzewski K, Strominger JL. The killer's kiss: the many functions of NK cell immunological synapses. *Curr Opin Cell Biol* 2008;20:597-605.
- Orange JS. Formation and function of the lytic NK-cell immunological synapse. *Nat Rev Immunol* 2008;8:713-25.
- Krzewski K, Coligan JE. Human NK cell lytic granules and regulation of their exocytosis. *Front Immunol* 2012;3:335.
- Wood SM, Ljunggren HG, Bryceson YT. Insights into NK cell biology from human genetics and disease associations. *Cell Mol Life Sci* 2011;68:3479-93.
- Haliotis T, Roder J, Klein M, Ortaldo J, Fauci AS, Herberman RB. Chediak-Higashi gene in humans I. Impairment of natural-killer function. *J Exp Med* 1980;151:1039-48.
- Klein M, Roder J, Haliotis T, Korec S, Jett JR, Herberman RB, et al. Chediak-Higashi gene in humans. II. The selectivity of the defect in natural-killer and antibody-dependent cell-mediated cytotoxicity function. *J Exp Med* 1980;151:1049-58.
- Abo T, Roder JC, Abo W, Cooper MD, Balch CM. Natural killer (HNK-1+) cells in Chediak-Higashi patients are present in normal numbers but are abnormal in function and morphology. *J Clin Invest* 1982;70:193-7.
- Stinchcombe JC, Page LJ, Griffiths GM. Secretory lysosome biogenesis in cytotoxic T lymphocytes from normal and Chediak-Higashi syndrome patients. *Traffic* 2000;1:435-44.
- Bhambhani V, Introne WJ, Lungu C, Cullinane A, Toro C. Chediak-Higashi syndrome presenting as young-onset levodopa-responsive parkinsonism. *Mov Disord* 2013;28:127-9.
- Weisfeld-Adams JD, Mehta L, Rucker JC, Dembitzer FR, Szporn A, Lublin FD, et al. Atypical Chediak-Higashi syndrome with attenuated phenotype: three adult siblings homozygous for a novel *LYST* deletion and with neurodegenerative disease. *Orphanet J Rare Dis* 2013;8:46.
- Krzewski K, Gil-Krzewska A, Nguyen V, Peruzzi G, Coligan JE. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 2013;121:4672-83.
- Whiteside TL. Measurement of cytotoxic activity of NK/LAK cells. *Curr Protoc Immunol* 2001;Chapter 7, Unit 7.18: 7.18.1-7.18.13.
- Mentlik AN, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Mol Biol Cell* 2010;21:2241-56.
- Sibarita JB. Deconvolution microscopy. *Adv Biochem Eng Biotechnol* 2005;95:201-43.
- Brown AC, Oddos S, Dobbie IM, Alakoskela JM, Parton RM, Eissmann P, et al. Remodelling of cortical actin where lytic granules dock at natural killer cell immune synapses revealed by super-resolution microscopy. *PLoS Biol* 2011;9:e1001152.
- Rak GD, Mace EM, Banerjee PP, Svitkina T, Orange JS. Natural killer cell lytic granule secretion occurs through a pervasive actin network at the immune synapse. *PLoS Biol* 2011;9:e1001151.
- Wood SM, Meeths M, Chiang SC, Bechensteen AG, Boelens JJ, Heilmann C, et al. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. *Blood* 2009;114:4117-27.
- Bryceson YT, Pende D, Maul-Pavicic A, Gilmour KC, Ufheil H, Vraetz T, et al. A prospective evaluation of degranulation assays in the rapid diagnosis of familial hemophagocytic syndromes. *Blood* 2012;119:2754-63.
- Bryceson YT, March ME, Ljunggren H-G, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 2006;107:159-66.
- Katz P, Zaytoun AM, Fauci AS. Deficiency of active natural killer cells in the Chediak-Higashi syndrome. Localization of the defect using a single cell cytotoxicity assay. *J Clin Invest* 1982;69:1231-8.
- Baetz K, Isaaz S, Griffiths GM. Loss of cytotoxic T lymphocyte function in Chediak-Higashi syndrome arises from a secretory defect that prevents lytic granule exocytosis. *J Immunol* 1995;154:6122-31.
- Jessen B, Maul-Pavicic A, Ufheil H, Vraetz T, Enders A, Lehmborg K, et al. Subtle differences in CTL cytotoxicity determine susceptibility to hemophagocytic lymphohistiocytosis in mice and humans with Chediak-Higashi syndrome. *Blood* 2011;118:4620-9.

38. Westbroek W, Adams D, Huizing M, Koshoffer A, Dorward H, Tinloy B, et al. Cellular defects in Chediak-Higashi syndrome correlate with the molecular genotype and clinical phenotype. *J Invest Dermatol* 2007;127:2674-7.
39. Striegl H, Andrade-Navarro MA, Heinemann U. Armadillo motifs involved in vesicular transport. *PLoS One* 2010;5:e8991.
40. Tewari R, Bailes E, Bunting KA, Coates JC. Armadillo-repeat protein functions: questions for little creatures. *Trends Cell Biol* 2010;20:470-81.
41. Klopfenstein DR, Vale RD. The lipid binding pleckstrin homology domain in UNC-104 kinesin is necessary for synaptic vesicle transport in *Caenorhabditis elegans*. *Mol Biol Cell* 2004;15:3729-39.
42. Scheffzek K, Welte S. Pleckstrin homology (PH) like domains—versatile modules in protein-protein interaction platforms. *FEBS Lett* 2012;586:2662-73.
43. Lemmon MA. Pleckstrin homology domains: not just for phosphoinositides. *Biochem Soc Trans* 2004;32:707-11.
44. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* 2009;10:513-25.
45. Krzewski K, Cullinane AR. Evidence for defective Rab GTPase-dependent cargo traffic in immune disorders. *Exp Cell Res* 2013;319:2360-7.
46. Kypri E, Falkenstein K, Lozanne AD. Antagonistic control of lysosomal fusion by Rab14 and the Lyst-related protein LvsB. *Traffic* 2013;14:599-609.
47. Smith TF, Gaitatzes C, Saxena K, Neer EJ. The WD repeat: a common architecture for diverse functions. *Trends Biochem Sci* 1999;24:181-5.
48. Liu D, Martina JA, Wu XS, Hammer JA 3rd, Long EO. Two modes of lytic granule fusion during degranulation by natural killer cells. *Immunol Cell Biol* 2011;89:728-38.
49. van der Sluijs P, Zibouche M, van Kerkhof P. Late steps in secretory lysosome exocytosis in cytotoxic lymphocytes. *Front Immunol* 2013;4:359.
50. Sepulveda FE, Burgess A, Heiligenstein X, Goudin N, Menager MM, Romao M, et al. LYST controls the biogenesis of the endosomal compartment required for secretory lysosome function. *Traffic* 2015;16:191-203.
51. Rozenszajn LA, David EB, Sela SB. Large granules and lysosomal fusion in human Chediak-Higashi white blood cells. *Acta Haematol* 1977;57:279-89.
52. Spicer SS, Sato A, Vincent R, Eguchi M, Poon KC. Lysosome enlargement in the Chediak-Higashi syndrome. *Fed Proc* 1981;40:1451-5.
53. Hammel I, Lagunoff D, Galli SJ. Regulation of secretory granule size by the precise generation and fusion of unit granules. *J Cell Mol Med* 2010;14:1904-16.
54. Perou CM, Leslie JD, Green W, Li L, Ward DM, Kaplan J. The Beige/Chediak-Higashi syndrome gene encodes a widely expressed cytosolic protein. *J Biol Chem* 1997;272:29790-4.
55. Durchfort N, Verhoef S, Vaughn MB, Shrestha R, Adam D, Kaplan J, et al. The enlarged lysosomes in beige jJcells result from decreased lysosome fission and not increased lysosome fusion. *Traffic* 2012;13:108-19.
56. Faigle W, Raposo G, Tenza D, Pinet V, Vogt AB, Kropshofer H, et al. Deficient peptide loading and MHC class II endosomal sorting in a human genetic immunodeficiency disease: the Chediak-Higashi syndrome. *J Cell Biol* 1998;141:1121-34.
57. Holland P, Torgersen ML, Sandvig K, Simonsen A. LYST affects lysosome size and quantity, but not trafficking or degradation through autophagy or endocytosis. *Traffic* 2014;15:1390-405.
58. Reefman E, Kay JG, Wood SM, Offenhauser C, Brown DL, Roy S, et al. Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. *J Immunol* 2010;184:4852-62.
59. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood* 2011;118:9-18.

SUPPLEMENTARY MATERIAL

Supplementary Methods

Antibodies:

The following Abs were used: CD3 (UCHT1), CD56 (HCD56), NKG2D (1D11), 2B4 (C1.7), CD11a (TS2/4), NKp44 (P44-8), NKp80 (5D12), KIR2DL1/S1 (HP-MA4), KIR2DL2/L3 (DX27), KIR3DL1 (DX9), ILT2 (GHI/75), NKR-P1A (HP-3G10), TNF α (MAb11), IFN γ (4S.B3), granzyme A (CB9), perforin (dG9), LAMP1 (H4A3), LAMP2 (H4B4) (BioLegend); perforin (B-D48) (Cell Sciences); CD16 (CB16) (eBioscience); CI-MPR (Abcam); NKp46 (9E2), CD94 (HP-3D9), EEA-1 (14/EEA1) (BD Biosciences); NKG2A (131411) (R&D Systems); EEA-1 (C45B10) (Cell Signaling); Rab27a (Proteintech Group). Anti-HER2 Ab was a gift from Dr. Q. Li (NCI/CCR, Bethesda, MD).

Subjects and healthy donors:

Subjects with CHS enrolled in protocol 00-HG-0153 (NCT00005917) and provided written informed consent. The protocol was approved by the Institutional Review Board of the National Human Genome Research Institute. CHS was considered based on clinical findings and confirmed by identification of giant inclusions within leucocytes on peripheral blood smear. Mutations in *LYST* were identified in all subjects, and were previously reported.^{E2-E4} The exception is patient B in whom only one mutation has been identified; protein expression studies confirm reduced *LYST* levels consistent with a diagnosis of CHS. Patient A:1 and A:2 (siblings): A1454D, Y1687X; patient B: V2651F; patient C:1 and C:2 (siblings): 3276 Δ N, 3277 Δ T; patient D: R503X, G3309S; patient E:1 and E:2 (siblings): R1104X, G3408R. Throughout the text, patient A:1, A:2, and B are referred to as patients with mutations within ARM/HEAT domain of *LYST*, while patient C:1, C:2, D, E:1, and E:2 are referred to as patients with mutations within *LYST* BEACH domain.

Voluntary healthy donors were recruited at the Department of Transfusion Medicine, NIH, with the donor's informed consent, in accordance with the Declaration of Helsinki.

Approximately 40 ml of peripheral blood was collected by venipuncture at room temperature in tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood

using the standard Ficoll-Paque method. NK cells were isolated from PBMCs using EasySep Human NK cell enrichment kits (StemCell Technologies) according to manufacturer's protocol, and cultured in X-vivo medium (Invitrogen) supplemented with 100U/ml of IL-2. IL-2 cultured NK cells were used in experiments, unless otherwise noted.

Cells:

Human B lymphoblastoid 721.221 cells, erythroleukemia K562 cells, and ovarian carcinoma SK-OV3 cells were grown in complete RPMI1640 medium.

Cytotoxicity assay:

NK cell cytotoxicity was evaluated by the DELFIA assay (Perkin-Elmer). Briefly, 721.221, K562 or SK-OV3 target cells were labeled with BATDA (Perkin Elmer) for 30 min at 37°C in complete RPMI1640 medium. Labeled target cells were transferred to 96-well polystyrene plates (U-bottom, Nunc), mixed with NK cells or NK cell lines (YTS, NK92mi) at different effector-to-target (E:T) ratios, and incubated for 2 h at 37°C. For ADCC, NK cells were incubated with SK-OV3 target cells in the presence of 50 ng/ml of either anti-HER2 or control IgG Ab. After cytolysis the cell supernatant, containing the released TDA ligand, was added to the Europium (Eu) solution to generate the fluorescent Eu-TDA chelate. The fluorescence of Eu-TDA was measured using Wallac 1420 counter (Perkin Elmer). The amount of released TDA ligand in cell supernatants was regarded as the experimental TDA release. Total TDA release was measured after complete lysis of target cells by 1% NP40. Lytic units were calculated as described in Whiteside et al.^{E5} Lysis percentage was determined using the following equation: (experimental TDA release – spontaneously released TDA)/(total TDA release – spontaneously released TDA). The lysis percentage was then plotted against E:T ratio.

Flow cytometry:

For the analysis of the cell surface receptors, NK cells were stained with the antibodies indicated in the text (Fig E2), and analyzed by multi-color flow cytometry. Compensation controls were set with the same antibodies used to stain the cells and the CompBead Plus kit (BD).

Delivery of granzyme B to 721.221 target cells was assessed using GranToxiLux assay kit (OncoImmunin). In this assay, target cells are labeled with a cell-permeable fluorogenic granzyme B substrate; upon delivery of granzyme B to the target cell the substrate is cleaved resulting in increased fluorescence in target cells.⁶ Briefly, target cells were labeled with TFL-4 (1:3000) and mixed with unlabeled effector cells at a ratio of 1:2. Immediately after mixing, fluorogenic granzyme B substrate was added to cells and the samples were incubated for 30 min at 37°C. Following incubation, cells were washed and analyzed by flow cytometry. The delivery of granzyme B was defined as the percentage of TFL-4-positive live cells (representing the target cell population) that were positive for fluorescence of the granzyme B substrate from the total pool of live TFL-4-positive target cells.

The data acquisition and analysis were done using FACSsort or LSRII cytometers (BD Biosciences), and FlowJo software (v. 10; Tree Star).

Cytokine production and release:

For analysis of total TNF- α or IFN- γ protein levels, 2×10^5 NK cells were first mixed with K562 target cells at 1:1 ratio for 0, 2, 4, 6, 12 or 18 h at 37°C. The cells were next incubated in 10% human serum to block Fc receptors, followed by staining with anti-CD56-APC antibody, fixation, and permeabilization with Cytofix/Cytoperm buffer (BD). Finally, the cells were stained with anti-TNF- α -PE or anti-IFN- γ -PE. The data acquisition and analysis were done using FACSsort cytometer (BD), and FlowJo software (v. 10; Tree Star).

To determine the ability of NK cells to secrete TNF- α or IFN- γ , 0.5×10^6 cells were either left unstimulated or stimulated for 20 h with IL-12 (20 ng/ml), IL-15 (100 ng/ml) and IL-18 (100 ng/ml). The amount of cytokines released to the cell culture supernatant, or in blood sera obtained from healthy donors or CHS patients, was determined using the Human TNF- α or IFN- γ ELISA MAXTM Deluxe kit (BioLegend),

according to the manufacturer's protocol. Calibration curves were prepared using the recombinant human TNF- α or IFN- γ , supplied with the kits.

Cell conjugation:

2×10^5 NK cells and 4×10^5 721.221 cells were labeled, respectively, with 2 μ M CMFDA (Invitrogen) and TFL-4 (1:2000). Cells were washed three times in PBS with 2% FCS and mixed together at an E:T ratio of 1:2. Cells were immediately transferred to 37°C and incubated for 15 or 30 min, followed by fixation with 1% paraformaldehyde and analysis by flow cytometry. For time 0, the cells were mixed and immediately fixed. The conjugate percentages were established by determination of CMFDA+ TFL-4+ double positive cells, representing NK cells conjugated with target cells, from the total pool of live CMFDA-positive NK cells.

Microscopy and image analysis:

NK cells were left alone, or mixed with 721.221 target cells at a 1:1 ratio for 20 min at 37°C in complete RPMI, followed by adherence to Excell Adhesion slides (EMS) for 10 min at 37°C. Cells were fixed and permeabilized with Cytofix/Cytoperm buffer with 0.1% Triton X-100, and blocked with 1% BSA. The cells were stained with anti-LAMP1 or -LAMP2 Ab followed by AlexaFluor 647-conjugated IgG1-specific anti-mouse Ab, anti-pericentrin followed by AlexaFluor 568-conjugated anti-rabbit Ab, or AlexaFluor 568-conjugated phalloidin, blocked with 5% normal mouse serum and then stained with AlexaFluor 488-conjugated anti-perforin Ab. In some experiments NK cells were stained with AlexaFluor 488-conjugated anti-perforin Ab, blocked with 5% normal mouse serum and then stained with AlexaFluor 647-conjugated anti-granzyme A Ab. In the experiments determining the location of vesicular compartments, the fixed and permeabilized cells were stained with AlexaFluor 647-conjugated anti-perforin Ab, then anti-CI-MPR, -EEA-1, or -Rab27a Ab followed by AlexaFluor 568-conjugated anti-rabbit Ab, blocked with 5% normal mouse serum and then stained with anti-LAMP1 Ab followed by AlexaFluor 488-conjugated anti-mouse Ab.

629 For staining with anti-MPR and EEA-1 Abs, the cells were fixed and stained first with anti-CI-MPR Ab
630 followed by AlexaFluor 568-conjugated anti-rabbit Ab, and then stained with anti-EEA1 Ab followed by
631 AlexaFluor 488-conjugated anti-mouse Ab. The cells were then blocked with 5% normal mouse serum
632 and stained with AlexaFluor 647-conjugated dG9 anti-perforin Ab.

633 For staining of cytokine-positive compartments, NK cells were stimulated with IL-12 and IL-18 for 6 h at
634 37°C, fixed and stained with anti-IFN- γ Ab followed by AlexaFluor 488-conjugated anti-mouse Ab, blocked
635 with 5% normal mouse serum, and stained with AlexaFluor 647-conjugated dG9 anti-perforin Ab. Cells,
636 mounted in ProLong Gold medium, were visualized by a Zeiss LSM710 Axiovert 200M laser-scanning
637 confocal microscope at room temperature. The images were obtained using a 63x Zeiss Plan-
638 Apochromat objective and Zeiss Zen 2012 software. Images shown represent a single optical section.

639 The granules (perforin) were regarded as polarized if the two following conditions were met: (i) perforin
640 localized into a triangular area, with the tips of the triangle defined by the center of the cell and the edge
641 points of the cell-cell interface, and (ii) the MTOC (as determined by pericentrin staining) was adjacent to
642 the cell-cell contact site, as described previously.^{E1} Co-localization was assessed by analysis of
643 overlapping pixels occupied by two fluorophores, using Imaris software (v. 7.6; Bitplane). A threshold $\geq$
644 25% than background fluorescence was used to determine the area occupied by each fluorophore and to
645 calculate the percentage co-localization, as described previously.^{E1} For co-localization between perforin
646 and LAMP1/LAMP2, the data were determined by analysis of the following number of cells: n = 30 - 45 for
647 healthy donors, n = 29 - 39 for CHS patients, and n = 8 - 10 for the transplanted CHS patient. For the co-
648 localization between perforin and different vesicular markers, the data were determined by analysis of the
649 following number of cells: n = 25 - 45 for CI-MPR, n = 8 - 11 for Rab27a, and n = 25 - 65 for EEA-1.

650 To assess the size and amount of lytic granules, NK cells were labeled for 30 min with 200 nM
651 LysoTracker Red DND-99 (Invitrogen), transferred to polylysine-coated Lab-Tek chambered cover glass
652 (Nunc) in complete X-Vivo medium, and placed in the Live Cell heated chamber (Pathology Devices).
653 Cells were allowed to settle for 1 min and then imaged in all three planes (x, y, z) at 37°C using an
654 Olympus IX81 spinning-disk confocal microscope with 100x Olympus PlanApo objective (heated to 37°C).
655 Image acquisition was performed with MetaMorph software (v. 7.7.3; Molecular Devices) using the
656 streamlining function with the following parameters: 0.2 μ m z-axial dimension, 100 ms exposure per

657 frame, 25-35 frames per image stack. Next, the acquired images were deconvolved using Huygens
658 software (v. 4.4; Scientific Volume Imaging B.V.), to correct image degradation due to point spread
659 function (PSF).^{E7} PSF was obtained through distilling an experimental PSF from spherical bead images.
660 To obtain calibration images of beads, sub-resolution beads from the PS-Speck Microscope Point Source
661 Kit (Invitrogen) were placed in the polylysine-coated Lab-Tek chambered cover glass, allowed to settle for
662 5 min, and imaged as described above, with the z-axial dimension of 0.1 μm ; the PSF was reconstructed
663 from the averaged bead images using Huygens software, according to Scientific Volume Imaging protocol
664 (<http://www.svi.nl/RecordingBeads>). After obtaining the PSF, the iterative Maximum Likelihood Estimation
665 CMLE algorithm was used to effectively solve image convolution and perform deconvolution.
666 Deconvolved images were analyzed using Imaris. To determine the diameter and number of lytic
667 granules the Spots function was used, with the following parameters: estimated diameter for the vesicle
668 identification was set to 0.25 μm , spots were classified based on the Quality Criterion algorithm, Region
669 Growing function was based on the absolute fluorescence intensity, and Spot Diameter was determined
670 from Region border. The quantity of vesicles was derived from analysis of 70 and 99 cells in, respectively,
671 healthy donors and CHS patients (47 cells for ARM/HEAT domain mutation group, 52 cells for BEACH
672 domain mutation group). The vesicle size was determined from measurements of ~2900 (healthy donors)
673 and ~1100 vesicles (CHS patients; ARM/HEAT domain mutation group: 911 vesicles, BEACH domain
674 mutation group: 1077 vesicles).

675 The granule distances to the IS and MTOC, were estimated based on a previously described method,^{E8}
676 using Imaris software. To measure the distance between the MTOC and granules, the xy coordinates of
677 the MTOC and the granules (or the entire region outlined by lytic granules, if the centroids of individual
678 granules could not be determined) in the plane of MTOC were obtained, followed by calculation of the
679 length of the shortest line connecting the MTOC and granule centroids. To determine granule distance to
680 the IS, lines representing the shortest distance connecting the granule centroids with the F-actin
681 accumulated at the IS were obtained using Imaris software. Those data are shown in Fig E3. Since the
682 granule size differed among healthy donors and CHS patient groups, the measurements were normalized
683 to the average granule size in a particular NK cell group, as determined in Fig 3 (i.e. 352 nm for healthy
684 NK cells, 560 nm for NK cells from CHS patients with LYST ARM/HEAT domain mutations, 397 nm for

NK cells from CHS patients with LYST BEACH domain mutations), according to the following equation:
distance to the IS (or the MTOC) (in μm) / average granule size in the tested group (in μm). This was done
to compensate for the limitations in granule proximity to the IS or the MTOC caused by the granule size;
those data are shown in Fig 4 as normalized distances between granules and the MTOC or the IS, and
are expressed in number of average granule diameters. The values were determined by evaluation of 24
- 35 conjugates for each indicated NK cell group.

RNAi constructs:

Vector-based shRNA, purchased from Sigma, were used to mediate knock-down of LYST in YTS and
NK92mi NK cells. The non-targeting shRNA (SHC002; Sigma) was used as a negative control (CTRL
RNAi). The shRNA constructs (in pLKO1.puro vector), verified by DNA sequencing, were transfected into
293T cells with the psPAX2 and pMD2.G helper plasmids (AddGene) using PolyJet (SignaGen). The
medium containing lentivirus particles was used to infect 2×10^6 YTS or NK92mi cells, in the presence of
10 $\mu\text{g/ml}$ protamine sulphate (Sigma). After 48 h, NK cells were transferred to medium containing
puromycin and maintained in the selection medium to generate a stable LYST knock-down cell lines.
LYST expression in the cell lines was monitored at the mRNA level by qPCR.

RNA isolation, RT-PCR, and qPCR:

Total RNA was isolated from cells using the RNAqueous-4PCR kit (Ambion), according to the
manufacturer's protocol, with the DNase treatment step. The isolated RNA (300 ng) was used to generate
cDNA, using qScript cDNA Synthesis Kits (Quanta Biosciences), according to the manufacturer's
suggestions. The generated cDNA served as template for real-time PCR, using SYBR Green I Master Mix
and LightCycler 480 (Roche), according to manufacturer's recommendations. Primers for real-time PCR
measurement of LYST and actin were purchased from Qiagen. All reactions were done in triplicates and
the average value of triplicates was used for calculating the relative levels of each mRNA species. Melting
curve analysis was carried out at the end of each real-time PCR run, to verify that only one product was
amplified. The mixture of cDNA from control and LYST RNAi cells was used to generate the standard

curve. The amount of the target gene mRNA was calculated from the standard curve, using the Roche LightCycler software, and normalized to actin mRNA.

Statistical analysis:

Statistical analysis was performed using one-way ANOVA (with Tukey's post-test), two-way ANOVA or unpaired t-test (GraphPad software). Alpha level was set to 0.05. Only statistically significant results are indicated in the figures.

Supplementary References

- E1. Krzewski K, Gil-Krzewska A, Nguyen V, Peruzzi G, Coligan JE. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood*. 2013;121(23):4672-4683. 10.1182/blood-2012-08-453738.
- E2. Karim MA, Suzuki K, Fukai K, et al. Apparent genotype-phenotype correlation in childhood, adolescent, and adult Chediak-Higashi syndrome. *Am J Med Genet*. 2002;108(1):16-22.
- E3. Bhambhani V, Introne WJ, Lungu C, Cullinane A, Toro C. Chediak-Higashi syndrome presenting as young-onset levodopa-responsive parkinsonism. *Mov Disord*. 2013;28(2):127-129. 10.1002/mds.25386.
- E4. Weisfeld-Adams JD, Mehta L, Rucker JC, et al. Atypical Chediak-Higashi syndrome with attenuated phenotype: three adult siblings homozygous for a novel LYST deletion and with neurodegenerative disease. *Orphanet J Rare Dis*. 2013;8:46. 10.1186/1750-1172-8-46.
- E5. Whiteside TL. Measurement of Cytotoxic Activity of NK/LAK Cells. Current Protocols in Immunology: John Wiley & Sons, Inc.; 2001.
- E6. Packard BZ, Telford WG, Komoriya A, Henkart PA. Granzyme B activity in target cells detects attack by cytotoxic lymphocytes. *Journal of immunology*. 2007;179(6):3812-3820. Prepublished on 2007/09/06 as DOI.
- E7. Sibarita JB. Deconvolution microscopy. *Adv Biochem Eng Biotechnol*. 2005;95:201-243.
- E8. Mentlik AN, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. *Molecular biology of the cell*. 2010;21(13):2241-2256. Prepublished on 2010/05/07 as DOI 10.1091/mbc.E09-11-0930.

Supplementary Figure Legends

Fig E1. Impaired cytolytic activity of CHS NK cells.

NK cells isolated from the indicated individuals, and cultured with IL-2, were mixed with 721.221, K562, or SKOV-3 target cells. Target cell lysis was determined as described in Supplementary Methods. The graphs summarize NK cell cytotoxicity data for the E:T ratios of 5:1 (*left*) and 1:1 (*right*), and show the values for individual patients with means $\pm$ SD. Statistical significance is indicated by asterisks; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired t test).

Fig E2. NK cells from CHS patients have normal levels of activating and inhibitory cell surface receptors.

PBMCs isolated from healthy donors or CHS patients were stained with anti-CD56 and anti-CD3 mAb, along with antibodies against the indicated receptors. The cells were then analyzed by flow cytometry. Lymphocyte population in the PBMC was gated first (based on forward and side scatter distribution), followed by gating on CD56⁺ CD3⁻ NK cells within the lymphocyte population. To determine the cell surface expression of NK cell receptors, the median fluorescence values for the indicated activating (**A**) or inhibitory receptors (**B**) were measured. The graphs show the values for individual persons, with means $\pm$ SD. There were no significant changes in the receptor expression on the surface of normal and CHS NK cells.

Fig E3. *LYST* silencing in NK cell lines reproduces findings from CHS NK cells.

A, YTS or NK92mi cells were transduced with control (CTRL) or *LYST* RNAi. Levels of *LYST* transcripts in the indicated cells were analyzed by real-time PCR. The graph shows relative expression of *LYST*, normalized to actin and calibrated to *LYST* levels in CTRL RNAi cells (value = 1). Data are represented as means $\pm$ standard deviation (SD) from 3 independent experiments.

B, Visualization of perforin in cells. YTS cells, transduced with CTRL or LYST RNAi were fixed and stained with anti-perforin Ab. Multiple optical sections were acquired every 0.3 μm in order to visualize all the perforin in the cell. The images show two-dimensional reconstruction of overlaid optical sections of the indicated transduced YTS cells. Inserts show DIC images of the cells. Scale bars represent 5 μm .

C, NK92mi cells transduced with CTRL or LYST RNAi were activated by mixing with 721.221 target cells for 30 min at 37°C. Following fixation, the cells were stained with Abs against perforin (green), pericentrin (MTOC marker; blue), and LAMP2 (red). The dashed lines show the position of the immunological synapses. Scale bars represent 5 μm . Inserts show DIC images; NK92mi cells are positioned on the top of target cells.

D, Cytotoxic activity of YTS and NK92mi cells, untransduced, or transduced with CTRL or LYST RNAi. The percentages of 721.221 target-cell lysis for different effector-to-target (E:T) ratios are shown as mean values + SD determined from at least 3 experiments.

Fig E4. Normal F-actin accumulation at the immunological synapse and altered co-localization between perforin and LAMP2 in CHS NK cells.

A, NK cells isolated from healthy donors or CHS patients, and cultured with IL-2, were activated by mixing with 721.221 target cells for 30 min at 37°C. The cells were next fixed, and then stained with Ab against perforin (green) and LAMP2 (red). F-actin was visualized by staining with phalloidin (blue). The cell outlines are indicated by short-dashed lines. The long-dashed lines show the position of the immunological synapses; the lines were omitted from the panels showing phalloidin staining to provide an unobstructed view of F-actin at the cell-cell contact site. The arrows point at the vesicles positive for perforin and negative for LAMP2; the arrowheads indicate the vesicles positive for LAMP2 and negative for perforin. Scale bars represent 5 μm . Two representative examples for each group are shown.

B, Perforin polarization to the immunological synapse following the interaction between NK and 721.221 target cells, as shown in **A**. Perforin was regarded as polarized if it localized to a conical area, limited by the center of the cell and the edge points of the cell-cell interface, and the MTOC (as determined by

pericentrin staining) was adjacent to the cell-cell contact site.^{E1} The graph shows the values for individual patients, with means $\pm$ SD. The values were determined by evaluation of at least 100 conjugates for each indicated group. ** $p < 0.01$, one-way ANOVA.

C, Perforin convergence to the MTOC and the immunological synapse following the interaction between NK and 721.221 target cells, as shown in A), and Fig 4A. The graphs illustrate the distances between perforin-positive granules and the MTOC (*top*), or the immunological synapse (*bottom*). The box extends from the 25th to 75th percentile, the line in the middle of the box indicates the median, “+” sign depicts the mean, whiskers show the range between the smallest and largest values. The values were determined by evaluation of 24 - 35 conjugates for each indicated NK cell group.

Fig E5. Enlarged granules in CHS NK cells are positive for perforin and granzyme A.

NK cells isolated from healthy donors, CHS patients or the transplanted CHS patient, and cultured with IL-2, were left unstimulated (**A**), or were activated by mixing with 721.221 target cells for 30 min at 37°C (**B**). The cells were next fixed, and stained with AlexaFluor 488-conjugated anti-perforin mAb (green) and AlexaFluor 647-conjugated anti-granzyme A mAb (red). The short-dashed lines show the cell outlines (**A**, **B**); the long-dashed line indicates the position of the immunological synapse (**B**). Scale bars represent 5 μm .

Fig E6. NK cells from CHS patient that received a bone marrow transplant show normal lytic granule morphology and polarization to the immunological synapse.

NK cells isolated from a healthy donor or CHS patient who underwent bone marrow transplantation were cultured with IL-2 and then activated by mixing with 721.221 target cells for 30 min at 37°C. The cells were fixed, and then stained with Ab against perforin (green) and either LAMP1 or LAMP2 (red). MTOC position was visualized by anti-pericentrin staining (blue; upper panels); F-actin was visualized by staining with phalloidin (blue; lower panels). The cell outlines are indicated by short-dashed lines. The long-dashed lines show the position of the immunological synapses; the lines were omitted from the panels

showing phalloidin staining, to show an unobstructed view of F-actin at the immunological synapse. Scale bars represent 5 μm .

Fig E7. Perforin-positive vesicles in CHS NK cells contain non-lysosomal markers.

NK cells isolated from healthy donors or CHS patients, and cultured with IL-2, were fixed, permeabilized, and stained with Abs against CI-MPR (green), EEA-1 (blue), and perforin (red). The cell outlines are indicated by short-dashed lines; inserts show DIC images. Scale bars represent 5 μm . Two or three representative examples for each group are shown.

Fig E8. Increased production of TNF- α and IFN- γ in CHS NK cells.

A, NK cells from healthy donors or CHS patients, cultured with IL-2, were mixed (E:T ratio 1:1) and co-cultured with K562 target cells for 6 h at 37°C. Following the incubation, the cells were stained with anti-CD56 Ab, fixed, permeabilized, stained with anti-IFN- γ or anti-TNF- α Ab, and analyzed by flow cytometry. The plots show a typical result, and illustrate the strategy used to determine the percentage of cytokine-positive NK cells (shown in Fig 7A).

B, Blood sera of healthy donors or CHS patients (A:1, A:2, C:1, C:2, E:1, E:2) were collected and analyzed for the presence IFN- γ (*left*) or TNF- α (*right*) by ELISA. Cell culture supernatants from healthy (circles) or CHS NK cells (A:1, E:2; squares) stimulated for 16 h with IL-12 (20 ng/ml) and IL-18 (100 ng/ml) were used as positive controls. The graphs show the values for individual persons, with means $\pm$ SD.

Table E1. Clinical and laboratory characteristics of CHS patients examined in the study:

	A:1	A:2	B	C:1	C:2	D	E:1	E:2
Ethnic origin	German/French	German/French	Mixed Northern European	Pakistan	Pakistan	African-American	Irish/Native American	Irish/Native American
Familial disease	Yes	Yes	No	Yes	Yes	No	Yes	Yes
Parental consanguinity	No	No	No	No	No	No	No	No
Gender	Male	Male	Female	Female	Male	Male	Male	Male
Allele 1	c.4361C>A p.Ala1454Asp	c.4361C>A Ala1454Asp	c.7951G>T p.Val2651Phe	c.9827_9832ATA CAA p.3276ΔN, 3277ΔT	9827_9832ATACA A 3276ΔN, 3277ΔT	c.1507C>T p.Arg503X	c.3310C>T p.Arg1104X	c.3310C>T p.Arg1104X
Allele 2	c.5061T>A p.Tyr1687X	c.5061T>A p.Tyr1687X	n/d	c.9827_9832ATA CAA p.3276ΔN, 3277ΔT	c.9827_9832ATA CAA p.3276ΔN, 3277ΔT	c.9925G>A p.Gly3309Ser	c.10222G>A p.Gly3408Arg	c.10222G>A p.Gly3408Arg
Age (years)	28	25	28	43	38	24	26	21
Pigmentary abnormalities	Fair, OCA	Fair, OCA	Mild OCA	Scattered hypopigmented macules, mild OCA	Scattered hypopigmented macules	Scattered hypo/hyperpigmented macules, mild OCA	Mild OCA	Mild OCA
Fever	No	No	No	No	No	No	No	No
Hemophagocytosis	No	No	No	No	No	No	No	No
Splenomegaly	Yes	Yes	No	No	No	Upper normal	Upper normal	No
Hepatomegaly	Upper normal	Yes	Yes	No	No	No	Upper normal	No
Hb (g/dL)	14.9	15.8	13.5	12.0	14.4	13.4	14.5	15.7
Neutrophils (10 ⁹ /L)	0.7	1.24	0.95	2.39	2.30	1.41	2.96	3.29
Platelets (10 ⁹ /L)	103	187	294	357	315	256	272	296
Triglycerides (mg/dL)	71	154	109	152	79	59	136	96
Fibrinogen (mg/dL)	305	286	381	410	388	425	487	333
Ferritin (μg/L)	66	59	141	82	97	18	139	160
Neurological manifestations [§]	Parkinsonism, Absent DTRs, UE/LE weakness, Ataxia	Absent DTRs, LE Weakness, LD	Absent DTRs, LD	Parkinsonism, LE weakness, LD	Parkinsonism, LD	Parkinsonism, Ataxia, Absent DTRs, LD	Ataxia, mild UE/LE weakness, Absent DTRs, LD	Ataxia, Absent DTRs, LD
Outcome	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive

[§] Reported at some point during the course of the disease; OCA: Ocular albinism; DTRs: Deep tendon reflexes; LE: Lower extremity; UE: Upper extremity; LD: Learning disability; CSF=cerebrospinal fluid.

Patients B, E:1 and E:2 carried novel missense mutations, whereas the other mutations has been described previously.^{6,24,25}
n/d=not determined (second mutation in patient B was not found despite numerous attempts)

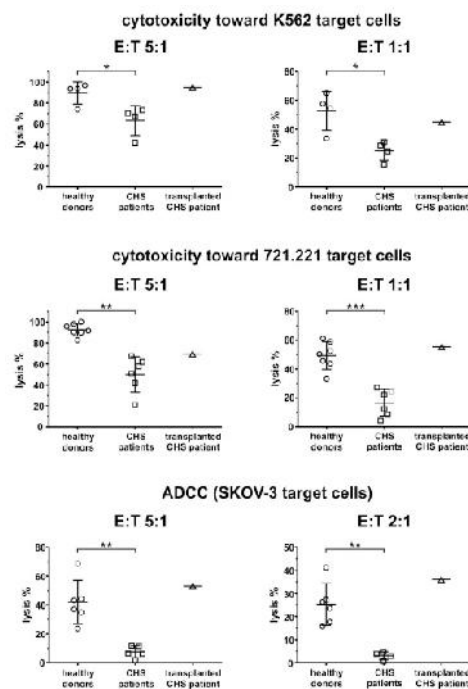


Fig E1.

Aleksandra Gil-Krzewska, Stephanie M. Wood, Yousuke Murakami, Victoria Nguyen, Samuel Cern Cher Chiang, Andrew R. Cullinane, Giovanna Peruzzi, William A. Gahl, John E. Coligan, Wendy J. Introne, Yen-an T. Bryceson, Konrad Krzewski

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<http://dx.doi.org/10.1016/j.jaci.2015.08.039>

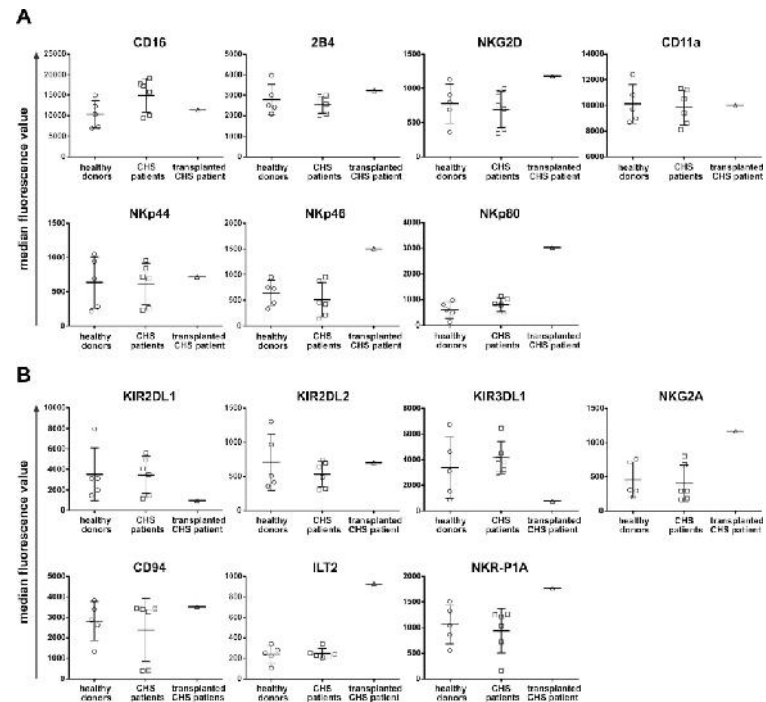


Fig E2.

Aleksandra Gil-Krzewska, Stephanie M. Wood, Yousuke Murakami, Victoria Nguyen, Samuel Cern Cher Chiang, Andrew R. Cullinane, Giovanna Peruzzi, William A. Gahl, John E. Coligan, Wendy J. Introne, Yen-an T. Bryceson, Konrad Krzewski

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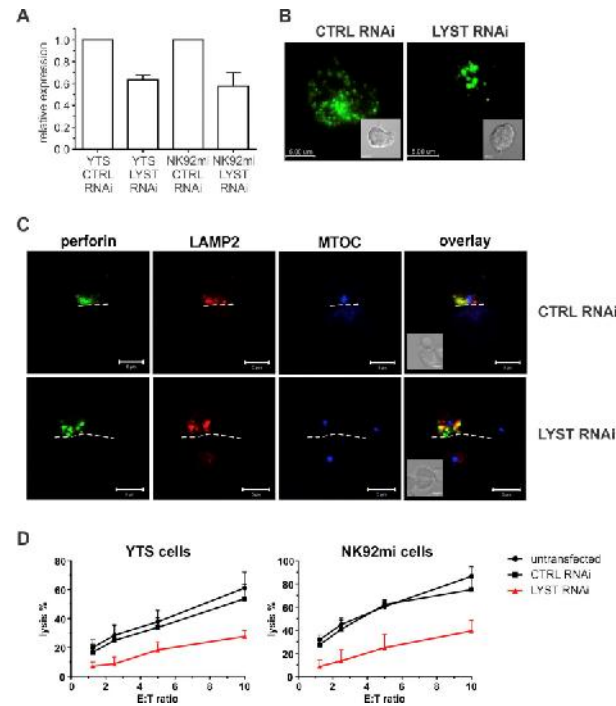


Fig E3.

Aleksandra Gil-Krzewska, Stephanie M. Wood, Yousuke Murakami, Victoria Nguyen, Samuel Cern Cher Chiang, Andrew R. Cullinane, Giovanna Peruzzi, William A. Gahl, John E. Coligan, Wendy J. Introne, Yenan T. Bryceson, Konrad Krzewski

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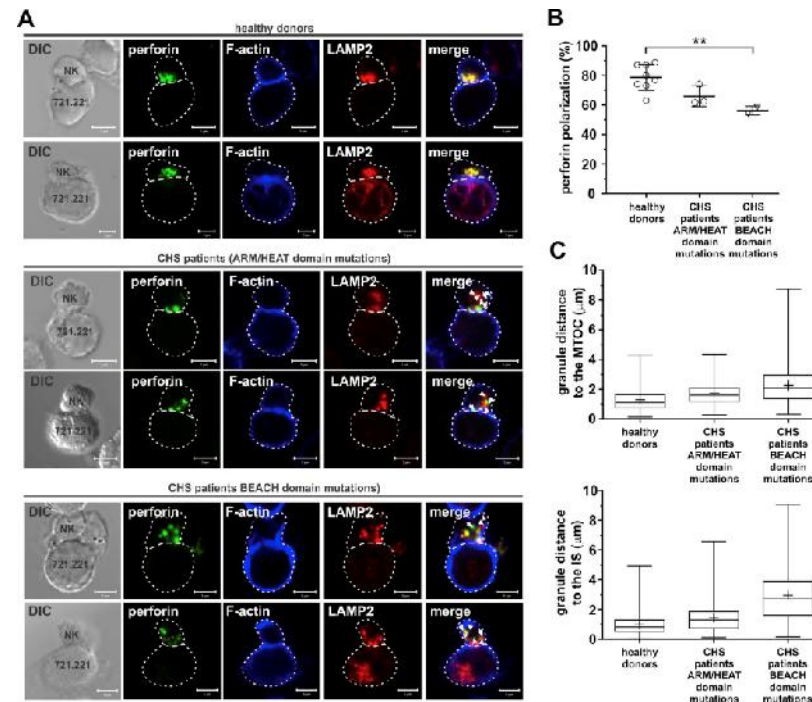


Fig E4.

Aleksandra Gil-Krzewska, Stephanie M. Wood, Yousuke Murakami, Victoria Nguyen, Samuel Cern Cher Chiang, Andrew R. Cullinane, Giovanna Peruzzi, William A. Gahl, John E. Coligan, Wendy J. Introne, Yenan T. Bryceon, Konrad Krzewski

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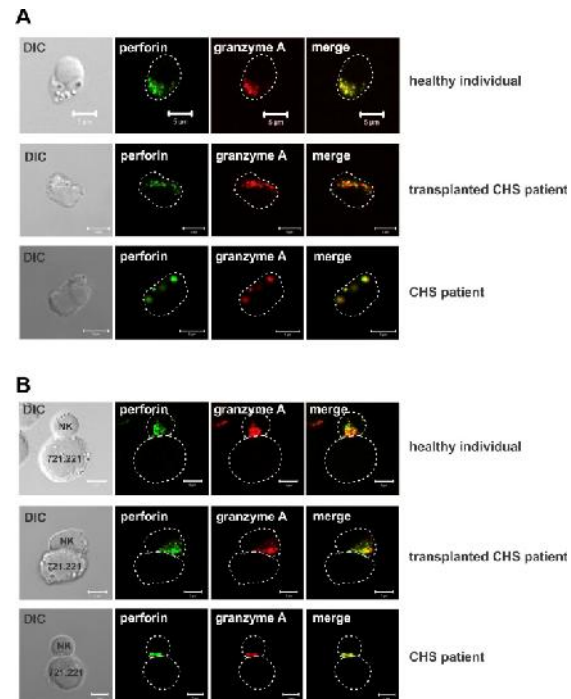


Fig E5.

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<http://dx.doi.org/10.1016/j.jaci.2015.08.039>

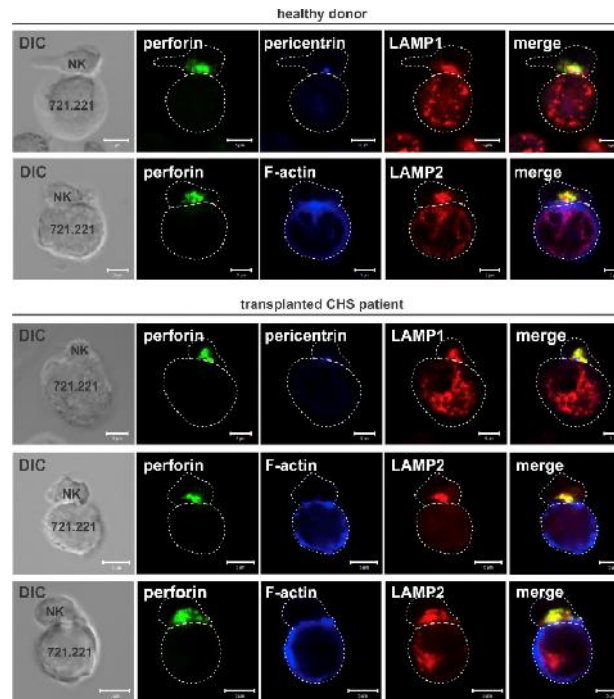


Fig E6.

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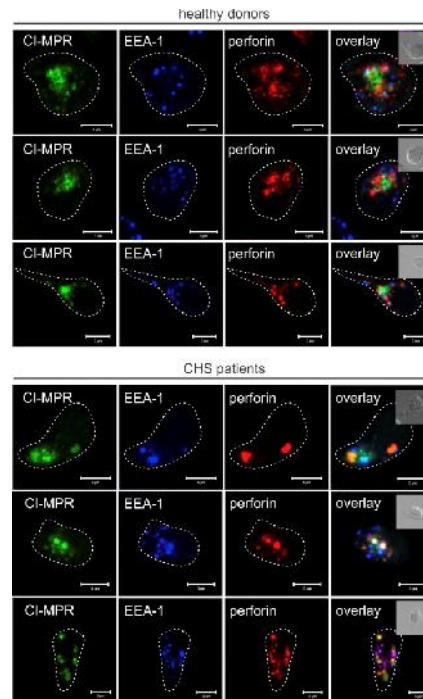


Fig E7.

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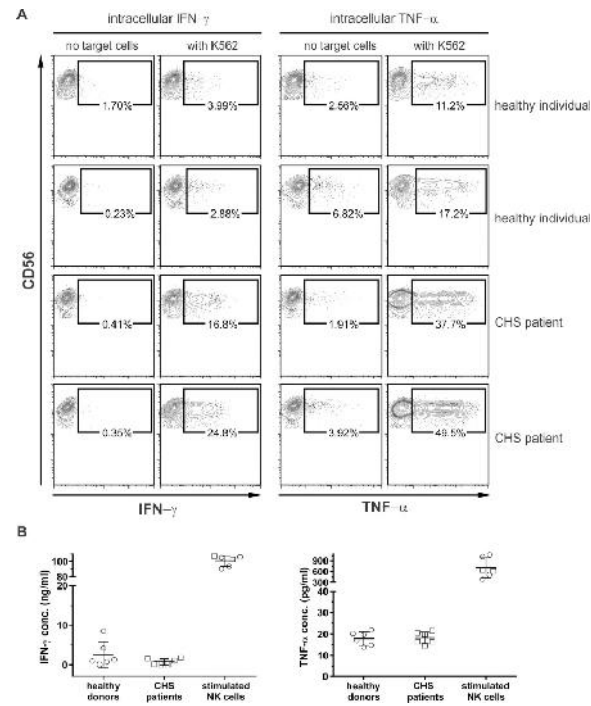


Fig E8.

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