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Chediak-Higashi syndrome: LYST domains regulate exocytosis of lytic granules, but not cytokine secretion by NK cells

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Abstract

Background—Mutations in *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 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 in CHS.

Methods—We analyzed NK cells from CHS patients with missense mutations in the LYST ARM/HEAT or BEACH domains.

Results—CHS NK cells 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 immunological synapse (IS); however, they were unable to properly fuse with the plasma membrane. Mutations in the BEACH domain resulted in

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AUTHORSHIP CONTRIBUTIONS

K.K., Y.T.B., A.G-K., and S.W. designed the study; A.G-K., K.K., S.W., Y.M., and V.N., G.P., and S.C.C.C. performed the experiments and acquired the data; W.J.I and W.A.G. recruited the patients and provided clinical information; A.R.C. performed the genetic analysis; K.K., Y.T.B., A.G-K., S.W., and W.J.I., analyzed and interpreted the data; K.K., Y.T.B., W.J.I., W.A.G. and J.E.C. wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURE

None of the authors has a conflicting interest. The authors declare no competing financial interests.

the formation of normal or slightly enlarged granules that had markedly impaired polarization to the immunological synapse, but could be exocytosed upon reaching the IS. Perforin-containing granules in CHS NK cells did not acquire certain lysosomal markers (LAMP1/2), but were positive for markers of transport vesicles (CI-MPR), late endosomes (Rab27a), and to some extent, early endosomes (EEA-1), indicating a lack of integrity in the endo-lysosomal compartments. CHS NK cells 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 the regulation of endo-lysosomal compartment identity. LYST functions in the regulated exocytosis, but not in the constitutive secretion pathway.

Keywords

Chediak-Higashi syndrome; LYST; NK cell; cytotoxicity; cytotoxic lymphocyte; lysosomes; lytic granules; exocytosis; immune deficiency

INTRODUCTION

Chediak-Higashi syndrome (CHS; OMIM 214500) is a rare lysosomal storage disorder, caused by mutations in *LYST*, which encodes the lysosomal trafficking regulator (LYST) protein.[1, 2] CHS is characterized by oculocutaneous albinism, bleeding diathesis, and immune dysregulation.[3-5] About 85–90% of patients develop 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 CHS patients develop a milder, attenuated form of the disease without HLH (atypical CHS).[6] A cell biologic 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, PH, BEACH and WD-40,[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 immuno-surveillance and host defense against cancer and microbial pathogens.[11] While 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 receptors,[15] and utilize their cytotoxic potential to eliminate abnormal cells and certain activated immune cells. Signals for activation and/or inhibition are generated at a specialized contact site formed between an NK cell and a target cell, known as the immunological synapse (IS).[16] Killing of target cells is a multi-stage 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 hemophagocytic lymphohistiocytosis type 2 – 5, Griscelli syndrome type 2 and CHS.[18, 19] NK cells in CHS patients have abnormal morphology and function; a study of two CHS patients 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 CHS NK cells,[22] and enlarged lytic granules have

been reported in cytotoxic T cells.[23] Nevertheless, our understanding of NK cell defects in 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

Please refer to the Supplementary Material in the Online Repository for detailed descriptions of methods and reagents.

Subjects and healthy donors

Subjects with CHS enrolled in protocol 00-HG-0153, approved by the NHGRI Institutional Review Board, and provided written informed consent. 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 for some of the cases (Table E1).[6, 24, 25] Voluntary healthy donors were recruited at the NIH, with informed consent, in accordance with the Declaration of Helsinki.

PBMCs were isolated from whole blood using Ficoll-Paque method, NK cells were isolated using EasySep Human NK cell kits (StemCell Technologies), and cultured in X-vivo medium supplemented with IL-2 (100U/ml). IL-2 cultured NK cells were used in experiments, unless otherwise noted.

Cytotoxicity assays

NK cell cytotoxicity was evaluated by the DELFIA assay (Perkin-Elmer) as described.[26] Lytic units were calculated as described previously.[27] Delivery of granzyme B to target cells was assessed as described.[26]

Cytokine production and release

For total cytokine levels, 2×10^5 NK cells were first mixed with K562 target cells at 1:1 ratio for the indicated times at 37°C. The cells were next stained with ant i-CD56-APC, fixed, permeabilized, and stained with anti-TNFα-PE or anti-IFNγ-PE. Data acquisition and analysis were done using FACSort and FlowJo.

Cytokine secretion was evaluated using the Human TNFα or IFNγ ELISA MAX™ Deluxe kit (BioLegend) after stimulating 0.5×10^6 cells for 20 h 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 min at 37°C, followed by adherence to Exce ll Adhesion slides (EMS) for 10 min at 37°C. Cells wer e fixed, permeabilized and stained with anti-LAMP1 or -LAMP2 Ab followed by AlexaFluor (AF)647-conjugated anti-mouse Ab, anti-pericentrin followed by AF568-conjugated anti-rabbit Ab, or AF568-conjugated phalloidin, and then stained with anti-perforin-AF488 Ab. In the experiments determining the location of vesicular compartments, fixed and

permeabilized cells were stained with anti-LAMP1 Ab followed by AF488-conjugated anti-mouse Ab, then anti-CI-MPR, -EEA-1, or -Rab27a Ab followed by AF568-conjugated anti-rabbit Ab, and stained with anti-perforin-AF647 Ab.

Cells, mounted in ProLong Gold, were visualized by a Zeiss LSM710 laser-scanning confocal microscope. The images were obtained using a 63x Zeiss Plan-Apochromat objective and Zeiss Zen software. Perforin polarization and co-localization was assessed as described previously.[26, 28]

To assess the size and amount of lytic granules, NK cells were labeled for 30 min with 200 nM LysoTracker, and transferred to polylysine-coated Lab-Tek chambered cover glass in complete X-Vivo medium. Cells were imaged in all three planes at 37°C using an Olympus IX81 spinning-disk confocal microscope with 100x Olympus PlanApo objective. Image acquisition was performed with MetaMorph software using the streamlining function with the following parameters: 0.2 μm z-axial dimension, 100 ms exposure per frame, 25-35 frames per image stack. To correct image degradation due to point spread function (PSF),[29] the acquired images were deconvolved using Huygens software with distilled experimental PSF. Deconvolved images were analyzed using Imaris and its Spots function.

RESULTS

Subject Characteristics

Eight subjects (6 males, 2 females age 21-43 years) from 5 families were studied (Table E1). Most patients carried compound heterozygous *LYST* mutations, with a 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 two amino acids in the BEACH domain of the protein. Patients from family A and B had mutations within the LYST ARM/HEAT domain, while patients from families C, D, and E had mutations within the LYST BEACH domain (Fig 1, Table E1 in the Online Repository). 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 2 takes prophylactic antibiotics for recurrent skin infections and patient 6 had bilateral osteomyelitis in childhood. To date, none of the patients has developed HLH. Throughout the text, based on *LYST* mutation positions, patient A:1, A:2, and B are referred to as patients with mutations within the LYST ARM/HEAT domain, while 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 female with CHS who received bone marrow transplant (BMT) at the age of 10; her samples were used as controls in the experiments.

Defective cytotoxicity of CHS NK cells

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

Next, we tested the cytotoxicity of NK cells isolated from patients suffering from atypical CHS. Compared to healthy donors, CHS patients had NK cells with significantly decreased natural cytotoxicity (against 721.221 and K562 cells), and almost completely inhibited antibody-dependent cell-mediated cytotoxicity (ADCC; against SK-OV3 cells) (Fig 2B). Inhibition of lytic activity was prominent for a wide range of effector-to-target ratios (Fig E1 in the Online Repository). The cytotoxicity was restored in NK cells from the CHS patient that received BMT. The decreased cytotoxicity was not due to impaired adhesion with target cells or modified levels of NK cell receptors, since CHS NK cells conjugated with target cells as normal NK cells (Fig 2C), and there was no significant difference in cell surface expression of several activating and inhibitory receptors between normal and CHS NK cells (Fig E2 in the Online Repository). Furthermore, silencing of *LYST* in NK cell lines also resulted in severe inhibition of cytotoxicity (Fig E8 in the Online Repository), confirming the importance of the gene for proper NK cell lytic function.

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

Since inhibition of CHS NK cell cytotoxicity 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 CHS NK cells. We asked 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 mean diameter); almost 50% of granules were smaller than 300 nm, and their size rarely exceeded 600 nm (Fig 3A), similar to former estimates.[30, 31] Most normal NK cells contained 30 to 60 granules (49±17;mean ±SD), with several cells containing up to 110 granules (Fig 3B), in line with previous data.[30] Normal NK cells accumulated filamentous (F-)actin (Fig E3 in the Online Repository), and polarized the microtubule organizing center (MTOC) toward the IS in response to target cell stimulation (Fig 4A). Lytic granules were positive for perforin and granzyme A (Fig E4 in the Online Repository), as well as for lysosomal markers, LAMP1 and LAMP2 (Fig 4A, Fig E3 in the Online Repository). Following target cell recognition, the majority of lytic granules polarized to the IS, forming tight clusters around the MTOC (Fig 4A, Fig E3B in the Online Repository). The average granule distance to the IS was 0.99±0.7 μm, which was equivalent to 2.8 diameters of normally-sized granules; the distance to the MTOC equaled 3.6 granule diameters (1.28±0.7 μm) (Fig 4B, Fig E3C in the Online Repository).

Granule distribution and morphology was different in CHS NK cells. Unexpectedly, instead of one huge granule as reported previously,[22] the size and the number of the lytic granules varied between NK cells from CHS patients with different mutations. NK cells from CHS patients with LYST mutations within the ARM/HEAT domain had markedly enlarged vesicles that were reduced in number. An average size of granule was increased to 560 nm, and almost 40% of the granules reached more than 600 nm in diameter (Fig 3A). The average number of granules was 19 (±8; SD), and ~50% of the NK cells had fewer than 20 granules, which was not observed in normal NK cells (Fig 3B). The large granules were positive for perforin and granzyme A and polarized to the IS upon NK cell binding to

susceptible target cells (Fig 4A-B, Fig E4 in the Online Repository). The granule distance to the IS was 1.45±0.9 μm, which was equivalent to 2.6 diameters of these large granules; the distance to the MTOC equaled 3 granule diameters (1.72±0.7 μm) (Fig 4B, Fig E3C in the Online Repository). In contrast, NK cells from CHS patients 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 percentage (~30%) of granules larger than 500 nm (Fig 3A). The majority of NK cells had between 15 and 30 granules (23±8; mean±SD). Interestingly, despite normal MTOC polarization to the IS in these NK cells, the granules did not translocate properly to the IS, as the distance to the IS increased to 2.96±1.7 μm, 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±1.2 μm) (Fig 4A-B, Fig E3C in the Online Repository).

Remarkably, we also observed striking differences in the location of lysosomal markers. Contrary to normal NK cells, CHS NK cells had granules that were positive for perforin but negative for LAMP1 or LAMP2, and vice versa (Fig 4A, Fig E3 in the Online Repository). NK cells from patients with LYST ARM/HEAT as well as BEACH domain mutations had significantly decreased co-localization between perforin and LAMP1 or LAMP2 (Fig 4C). CHS NK cells accumulated F-actin at the IS (Fig E3 in the Online Repository) which, combined with unaffected MTOC polarization to the IS (Fig 4), indicated normal CHS NK cell activation. Moreover, as in CHS NK cells, 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 (Fig E8 in the Online Repository). The CHS patient that underwent BMT had NK cells with granules indistinguishable from NK cells isolated from healthy donors, as expected (Fig E5 in the Online Repository).

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

The altered co-localization between perforin and LAMP1/LAMP2 proteins indicated that the granules in CHS NK cells only partially acquired lysosomal markers. Therefore, we asked about the distribution of other vesicular markers in CHS NK cells. 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, Fig E6 in the Online Repository), in agreement with previous studies.[26, 32] In comparison, nearly all perforin-positive granules in CHS NK cells were positive for CI-MPR (Fig 5A, C, and Fig E6 in the Online Repository), and the majority of perforin-positive vesicles were also positive for Rab27a (Fig 5B-C). Moreover, many of the perforin granules also acquired EEA-1, albeit at low levels (Fig 5A, C, and Fig

E6 in the Online Repository). Thus, perforin-containing granules in CHS NK cells combine features of different endo-lysosomal compartments, revealing a mixed vesicular identity.

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

The alterations in lytic granules size or polarization, suggested that inhibition of cytotoxicity could be caused by problems with degranulation of CHS NK cells.[33] The fact that LAMP1 and LAMP2 locations were altered in regard to perforin granules in CHS NK cells (Fig 4, Fig E3 in the Online Repository) led to our concern that the standard LAMP1 externalization assay might not accurately reflect NK cell degranulation in CHS patients. 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 CHS NK cells were able to deliver granzyme to target cells, and there was a notable 2.3-fold decrease in granzyme B delivery (Fig 6A-B). Degranulation of CHS NK cells was not totally defective, and some CHS NK cells were able to release their lytic granules, likely accounting for the observed residual cytotoxicity. While there was no difference in the percentage of target cells that received granzyme from NK cells with either *LYST* ARM/HEAT or 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 CHS patients with *LYST* ARM/HEAT domain mutations that had degranulated, delivered minimal amounts of granzyme B (Fig 6C); however, CHS NK cells with LYST BEACH domain mutations that were capable of degranulation, delivered granzyme amounts similar to normal NK cells (Fig 6C). There was no decrease in the amount of perforin or granzyme B in CHS NK cells when compared to normal NK cells (data not shown). The delivery of granzyme B from NK cells of CHS patient that received a bone marrow transplant was the same as from normal NK cells, indicating that these NK cells had normal cytotoxic potential.

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

Cytokine production and secretion in CHS NK cells

NK cells are potent producers of several cytokines, including TNF-α and IFN-γ.[14, 34] Since CHS NK cells showed defective lytic granule polarization or release, we asked 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 CHS NK cells, compared to normal NK cells (Fig 7A, Fig E7A in the Online Repository). The increased percentage of cytokine-producing CHS NK cells suggested a partial block of exocytosis (due to enlarged cytokine-positive granules) and/or differences in CHS NK cells response to stimulation. CHS NK cells secreted similar amounts of IFN-γ and TNF-α as normal NK cells in response to cytokine or target cell stimulation (Fig 7B 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 CHS and normal NK cells, and there was no fusion between perforin and IFN-γ granules (Fig 7C). Thus, the increased amount of cytokine-producing NK cells in CHS was not due to mixed compartmentalization or retention of cytokine vesicles. We found, however, that CHS NK cells responded faster to target cell stimulation than normal NK cells. There was no difference in cytokine production between CHS and normal NK cells immediately after mixing with target cells; nevertheless, within 2–3 h of stimulation the percentage of cytokine-producing CHS NK cells increased significantly compared to normal NK cells, and the amount of TNF-α- and IFN-γ- positive CHS NK cells peaked at 4 and 6 h of stimulation, respectively. Within 12–18 h, however, normal NK cells reach similar percentage of cytokine producing cells (Fig 7D), which could explain the lack of an overt difference in the amount of cytokine secreted by normal and CHS NK cells 20 h after stimulation (Fig 7B). Of note, CHS NK cells did not spontaneously secret TNF-α or IFN-γ (data not shown), and TNF-α and IFN-γ levels in sera of CHS patients were minimal and the same as the levels in sera of healthy donors (Fig E7B in the Online Repository). In summary, while *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, caused by mutations in the *LYST* gene, is characterized by formation of giant lysosomes and defects in lysosomal cargo trafficking and delivery. The disruption of vesicular traffic in Chediak-Higashi syndrome 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, Fig E1 in the Online Repository) or T cells in CHS has been reported before,[20-22, 35-37] but the majority of reports have described a single case with one or two classically affected individuals.[20, 21, 35, 36] Our study on a group of atypical CHS patients provides several important insights into mechanisms underlying defective cytotoxicity of NK cells in 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: ARM/HEAT, PH, BEACH and WD-40[2, 5, 9, 10] (Fig 1), but the function of individual LYST domains has not been elucidated. Uniquely, our data indicate that a correlation exists among granule size, their number and the location of *LYST* mutations. Missense mutations within the *LYST* BEACH domain lead to the formation of lytic granules with relatively normal size and number, while ARM/HEAT domain missense mutations result in granules of markedly increased size and decreased number (Fig 3). Previously, one giant lytic granule had been detected in CHS NK cells,[22] however the position of the LYST mutations was not determined in those cases; the fact that many of those CHS patients were children suggest that most likely 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 phenotype of classic and

atypical CHS patients could be quite different. Furthermore, the large granules in NK cells from CHS patients with *LYST* ARM/HEAT domain mutations are capable of translocation to the IS, while 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, Fig E3 in the Online Repository). 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, 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 two domains directly, and/or LYST conformation and domain positioning, resulting in absent or improper interactions between LYST and protein(s) 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* resulted in formation of giant lysosomes, reminiscent of those caused by mutation in *LYST* ortholog *LvsB*, whereas overexpression of a dominant negative form of Rab14 rescued the *LsvB* phenotype, restoring the size and morphology of post-lysosomal 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 may be less important for mediating vesicular fusion, but rather play an upstream role in granule movement (Fig 3-5). Another explanation would be that mutations in the BEACH domain change LYST conformation, resulting in miss-positioning of the WD-40 domain that 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, for example 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 CHS NK cells is currently not obtainable. Nevertheless, an altered protein stability, resulting from conformational changes and/or miss-folding, is a consequence of gene mutations. Thus, the conclusion about the relationship between the genotype and phenotype in CHS NK cells remains valid, and is supported by a consistent cellular phenotype observed in CHS patients with LYST ARM/HEAT or BEACH domain mutations.

We observed that only a portion of CHS NK cells was able to release their lytic granules and deliver granzyme B to target cells, indicating that defective lytic granule exocytosis (Fig 6 and Bryceson et al.[33]) 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 number observed in NK cells from CHS patients 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 due to increased granule size. The NK cell IS has been shown to contain a meshwork of filamentous actin, through which granules have to pass in order 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 clearances likely blocks the passage of such big vesicles and restricts their release. Indeed, CHS NK cells 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, CHS NK cells 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.

The defective exocytosis could also be due to miss-localization 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] While the majority of large perforin-positive granules are also positive for Rab27a in CHS NK cells, 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 degranulation potential of CHS T cells.[50] A very important finding is that the lytic granules in CHS NK cells display markers of usually distinct vesicular compartments. The nature of enlarged vesicles in CHS has been a matter of active debate, and two 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 due to deficient LYST function.[46, 50-53] The fission model maintains that LYST plays a role in vesicular fission and without LYST there is an incorrect separation of lysosomal membranes following normal fusion events.[23, 54, 55] Our data show that in CHS NK cells perforin-positive granules amass markers of several vesicular compartments, including proteins from late endosomes, transport vesicles, and even early endosomes (Fig 5, Fig E6 in the Online Repository). The acquisition of markers of trans-Golgi-derived transport vesicles and late endosomal proteins, as well as the presence of low levels of an early endosomal marker, EEA-1, suggests that the granules are formed through aberrant fusion of several vesicular compartments. Indeed, impaired integrity and/or maturation of endo-lysosomal compartments appears to be a common feature of CHS cytotoxic lymphocytes, as enlarged vesicles in CHS cytotoxic T cells 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 and CHS NK cells to the same extent (data not shown), indicating that pH of the granules in CHS NK cells is not affected, which agrees with reports showing that the acidification of large granules in CHS cells is not changed.[56, 57] We did not observe any difference in granzyme B activity between normal and CHS NK cells, and others have shown that processing and enzymatic activity of several lysosomal enzymes is not affected in CHS cells,[23, 57] implying that despite their mixed identity and enlarged size the granules are functional in 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 CHS NK cells (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 regulated exocytosis pathway, LYST is not required for the constitutive exocytosis pathway, which agrees with the ability of CHS patients to develop IFN-γ–driven hyper-inflammatory syndromes. Interestingly, we did not detect altered levels of TNF-α or IFN-γ in the sera of CHS patients examined in this study, suggesting that immune cells in CHS do not spontaneously secrete these cytokines, and likely require a trigger (e.g. a viral infection) to elicit their response and induce cytokine secretion, which when uncontrolled could lead to hyper-inflammation.[17]

CHS is undoubtedly a complex disease with many unanswered questions. For instance, is there a particular reason why the atypical CHS patients do not develop HLH? One conjecture would be that CHS cytotoxic lymphocytes retain some degree of functionality, and their partial lytic activity could prevent excessive accumulation of over-activated 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 CHS. In such a case, what is the risk for atypical CHS patients to develop HLH? Moreover, it is not clear to what extent *LYST* deficiency affects other cells of the immune system (e.g. dendritic cells, macrophages), production of pro- 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 patients with missense mutations affecting specific domains of the LYST protein we have gained novel insight into cellular phenotype of CHS NK cells. Our data show that CHS NK cells display very complex and heterogeneous range of defects related to lytic granule polarization, size and acquisition of endo-lysosomal markers, which likely provide a cumulative effect resulting in severely impaired NK cell cytotoxicity (Fig 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CHS	Chediak-Higashi syndrome
NK	Natural Killer (cell)
IS	immunological synapse
HLH	hemophagocytic lymphohistiocytosis
ADCC	antibody-dependent cell-mediated cytotoxicity
MTOC	microtubule organizing center
LYST	lysosomal trafficking regulator
LAMP1/2	lysosome-associated membrane protein
EEA1	early endosome antigen 1
CI-MPR	cation-independent mannose 6-phosphate receptor
Rab	Ras-associated binding protein

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KEY MESSAGES

1. LYST is involved in the regulation of cytotoxic granule size, movement and exocytosis, as well as in maintaining endo-lysosomal compartment integrity, but does do not control the size of cytokine compartments.
2. LYST mutations affect multiple aspects of NK cell cytotoxic activity, but not cytokine secretion.

CAPSULE SUMMARY

Chediak-Higashi syndrome NK cells display a complex cellular phenotype with heterogeneous range of defects related to lytic granule polarization, size and acquisition of endo-lysosomal markers that cumulatively result in severely impaired cytotoxicity without affecting cytokine secretion.

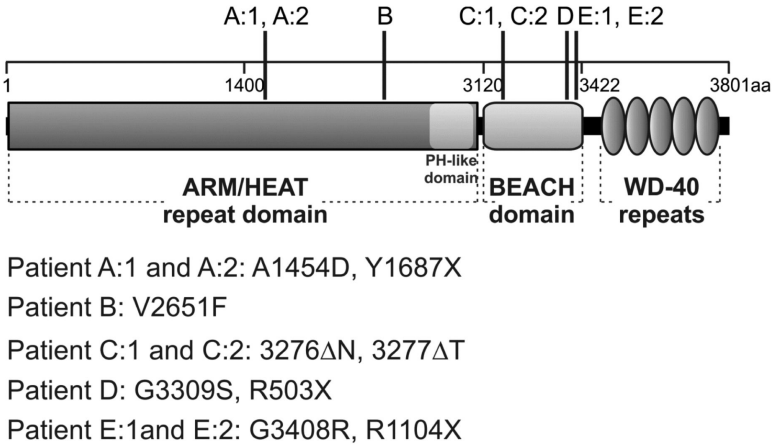

Figure 1. LYST and position of mutations in CHS patients examined in the study

Schematic representation of the domain organization of LYST. The positions of missense mutations identified in individual CHS patients included in this study (Table E1) are indicated. The type and location of the mutations in CHS patients are listed below the illustration.

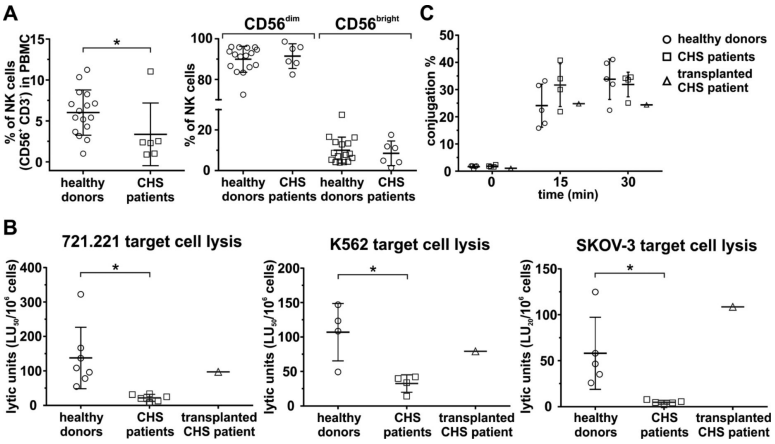

Figure 2. NK cells from Chediak-Higashi syndrome patients have significantly reduced natural and antibody-dependent cell-mediated cytotoxicity

A, The percentage of $CD3^{-}CD56^{+}$ NK cells in PBMCs, and the percentage of $CD56^{dim}$ and $CD56^{bright}$ cells in NK cell population of healthy donors and CHS patients.

B, Cytotoxic activity of NK cells against different cell lines.

C, The percentage of conjugate formation between NK and target cells.

The graphs show the values for individual patients, with means ± SD. * $p < 0.05$; only significant changes are indicated.

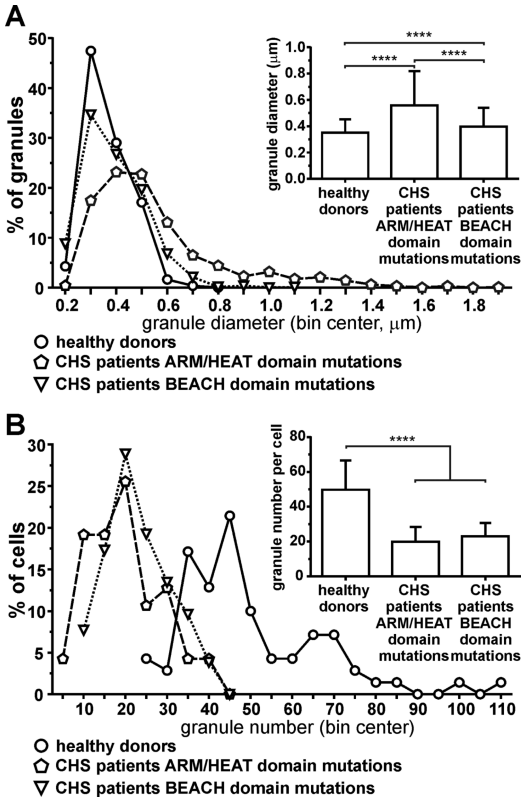

Figure 3. Distinct effects of *LYST* mutations on NK cell lytic granule size and quantity

NK cells from healthy donors or CHS patients, labeled with LysoTracker Red, were visualized using spinning disk confocal microscopy to determine the diameter (**A**) or number (**B**) of lytic granules. The line graphs illustrate the frequency distribution of granule size or amount; the inserted bar graphs show the mean values for the granule diameter or number; error bars indicate SD. **** $p < 0.0001$.

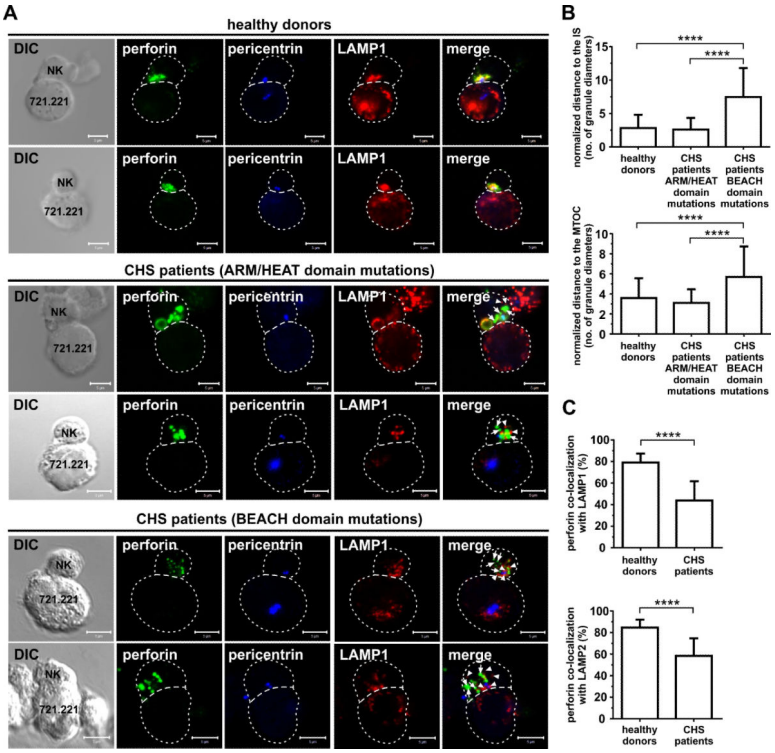

Figure 4. NK cells from Chediak-Higashi syndrome patients display a heterogeneous spectrum of defects related to lytic granule size, polarization and acquisition of lysosomal markers

A, NK cells isolated from healthy donors or the indicated CHS patients, mixed with target cells, were stained with Abs against perforin (green), pericentrin (MTOC marker; blue), and LAMP1 (red). Short-dashed lines indicate cell outlines; the long-dashed lines show the position of the immunological synapse. The arrows and arrowheads indicate the vesicles positive for perforin and negative for LAMP1 or vice-versa, respectively. Scale bars represent 5 μm. Two representative examples for each group are shown.

B, Perforin coalescence around the MTOC and polarization to the immunological synapse following the interaction between NK and target cells, as shown in **A**, and Fig E3 in the Online Repository. The graphs illustrate the mean values; error bars show SD. **** $p < 0.0001$.

C, The percentage of co-localization between perforin and LAMP1 or LAMP2. The data are shown as mean values + SD. * $p < 0.05$, **** $p < 0.0001$.

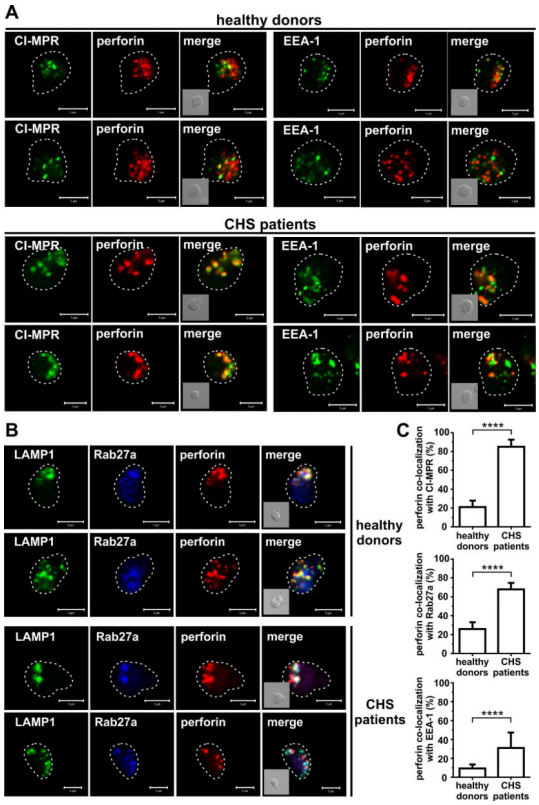

Figure 5. Perforin-containing granules of Chediak-Higashi syndrome NK cells are positive for markers of several different vesicular compartments

NK cells were stained with Abs against perforin (red) and different vesicular compartments: (**A**) CI-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; inserts show DIC images. Scale bars represent 5 μm. Two representative examples for each group are shown. There were no noticeable differences between patients with LYST ARM/HEAT or BEACH domain mutations.

C, The percentage of co-localization between perforin and CI-MPR, Rab27a, or EEA-1. The data are shown as mean values + SD. **** $p < 0.0001$.

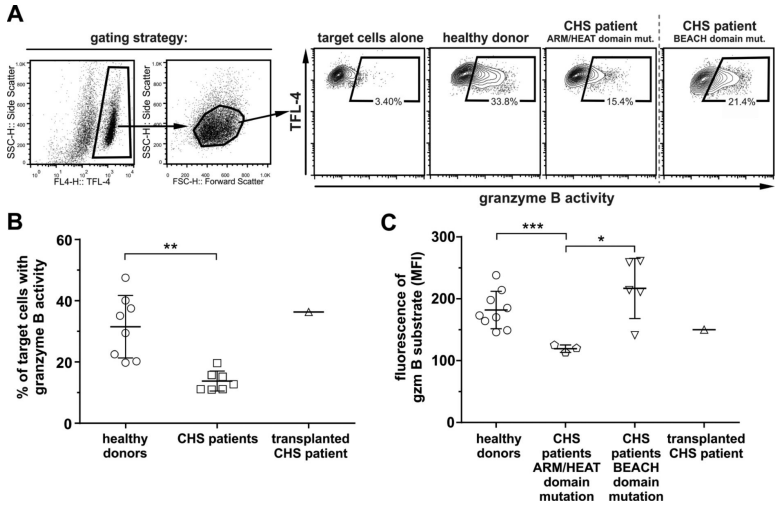

Figure 6. Impaired delivery of granzyme B from Chediak-Higashi syndrome NK cells to target cells

A, Granzyme B delivery from healthy or CHS NK cells to target cells. The plots show the gating strategy and illustrate representative results.

B, Quantification of the data shown in **A**. There were no differences between patients with LYST ARM/HEAT or BEACH domain mutations.

C, The target cells positive for granzyme B activity, as determined in **A**, were analyzed for the mean intensity fluorescence of granzyme B substrate.

The graphs in **B** and **C** show values for individual persons, with means ± SD. * $p < 0.05$, ** $p < 0.01$.

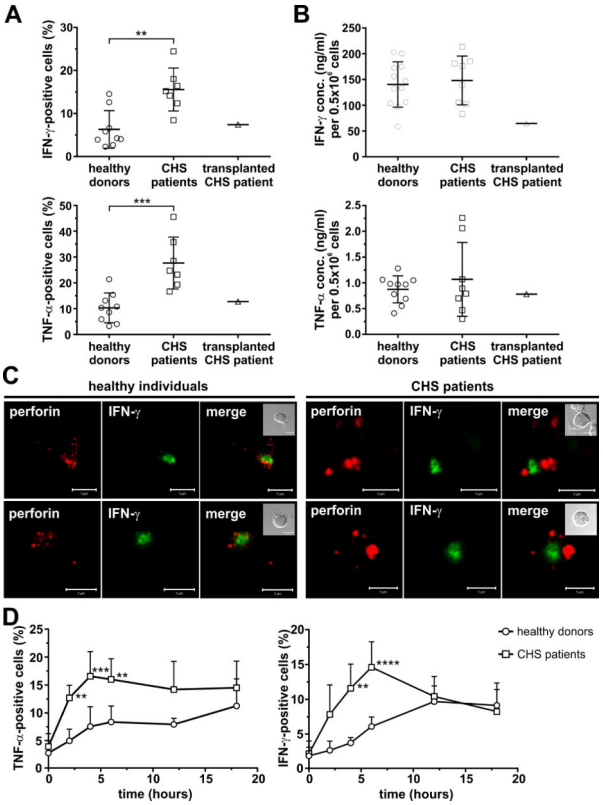

Figure 7. Chediak-Higashi syndrome NK cells produce cytokines in response to target cell stimulation faster than normal cells, and do not show evidence of a block in cytokine secretion or abnormal IFN-γ-positive compartments

A, IFN-γ and TNF-α production by NK cells, co-cultured with target cells. ** < 0.01, *** p < 0.001.

B, IFN-γ and TNF-α secretion by NK cells, stimulated with IL-12 and IL-18. The graphs in A-B show the values for individual persons, with means ± SD.

C, NK cells, stimulated with IL-12 and IL-18, were stained with Ab against perforin (red) and IFNγ (green). Scale bars represent 5 μm; inserts show the DIC images.

D, Time course of IFN-γ and TNF-α production by NK cells. Error bars represent SD; ** < 0.01, *** < 0.001, **** < 0.0001; only significant values are indicated.

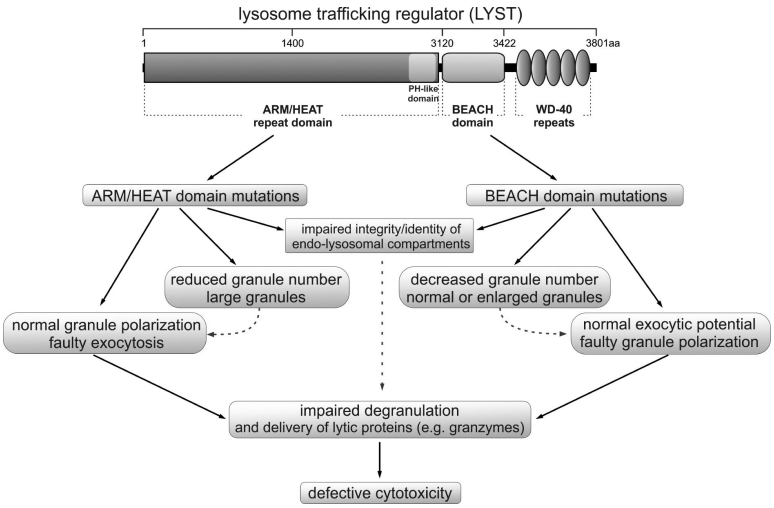

Figure 8. Association of mutation positions with multiple roles LYST plays in regulated exocytosis of NK cells

The diagram illustrates a schematic summary of the findings in patients with atypical CHS. Mutations within LYST ARM/HEAT domain lead to generation of large granules with severely reduced granule numbers. The 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 CHS patients with LYST ARM/HEAT domain mutations. On the other hand, mutations within LYST BEACH domain result in generation of decreased amount of granules that have normal or slightly enlarged size. While 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 CHS patients with LYST BEACH domain mutations, defective cytotoxicity results from inability of NK cells to properly translocate lytic granules to the contact site with target cells.

Therefore, while mutations in different domains of LYST produce different cellular phenotypes, they all result in common outcomes, such as a compromised integrity of the endo-lysosomal compartments and impaired granule exocytosis, ultimately leading to defective delivery of lytic proteins to the target cells and impaired cytotoxicity.