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Syntaxin 11 marks a distinct intracellular compartment recruited to the immunological synapse of NK cells to colocalize with cytotoxic granules

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Abstract

The *syntaxin 11* (*STX11*) gene is mutated in a proportion of patients with familial haemophagocytic lymphohistiocytosis (FHL) and exocytosis of cytotoxic granules is impaired in STX11-deficient NK cells. However, the subcellular localization, regulation of expression and molecular function of STX11 in NK cells and other cytotoxic lymphocytes remain unknown. Here we demonstrate that STX11 expression is strictly controlled by several mechanisms in a cell-type-specific manner and that the enzymatic activity of the proteasome is required for STX11 expression in NK cells. In resting NKL cells, STX11 was localized in the cation-dependent mannose-6-phosphate receptor (CD-M6PR)-containing compartment, which was clearly distinct from cytotoxic granules or Rab27a-expressing vesicles. These subcellular structures appeared to fuse at the contact area with NK-sensitive target cells as demonstrated by partial colocalization of STX11 with perforin and Rab27a. Although STX11-deficent allo-specific cytotoxic T-lymphocytes efficiently lysed target cells and released cytotoxic granules, they exhibited a significantly lower extent of spontaneous association of perforin with Rab27a as compared with STX11-expressing T cells. Thus, our results suggest that STX11 promotes the fusion of Rab27a-expressing vesicles with cytotoxic granules and reveal an additional level of complexity in the spatial/temporal segregation of subcellular structures participating in the process of granule-mediated cytotoxicity.

Keywords: lymphocyte • activation • cytotoxicity • degranulation • exocytosis

Introduction

Familial hemophagocytic lymphohistiocytosis (FHL) is a rare genetic disease, which typically manifests in infancy as rapidly developing systemic fatal inflammation (reviewed in Refs. [1–3]). Mutations in the genes encoding perforin (*PRF1*), Munc13-4 (*UNC13D*), syntaxin 11 *(STX11)* and syntaxin-binding protein 2 (*STXBP2)* have been linked to FHL cases categorized as FHL2, FHL3, FHL4 and FHL5, respectively [4–8]. All these genes are involved in the cytolytic activity of NK cells and/or cytotoxic T-lymphocytes (CTLs) that is mediated through exocytosis of specialized granules containing perforin and granzymes. FHL is believed to result from the impaired capacity of NK cells and CTLs to eliminate their targets, which consequently leads to lymphocyte hyperactivation and abnormally increased production of pro-inflammatory lymphokines. Perforin is required for delivery of

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granzymes to target cells, whereas Munc13-4 has been suggested to prime cytotoxic granules for fusion with the cell membrane after their docking at the immunological synapse, that is the contact area between the cytotoxic cell and its target [5,7]. STX11 and its recently identified binding partner STXBP2/Munc18-2 [4,9] are believed to regulate a fusion event occurring in the course of cytotoxic granule maturation and release. STX11 is a member of a family of membrane trafficking proteins referred to as soluble *N*-ethylmaliemide-sensitive factor attachment protein receptors and is known as a 35 kD binding partner of the ubiquitous SNARE SNAP-23. STX11 is expressed in different organs and in spite of the lack of a transmembrane domain behaves as a classical integral membrane protein associated with late endosomes and trans-Golgi network in HELA cells [10]. However, the exact molecular function of STX11 remains unknown.

Cytotoxic granule release was long viewed as a single membrane fusion event involving one vesicular structure and the plasma cell membrane. However, it has been recently demonstrated that Munc13-4 is segregated from cytotoxic granules in a different vesicular compartment [11], which also contains Rab27a, a small GTPase, whose deficiency is associated with Griscelli syndrome characterized by FHL-like disorder and depigmentation of hair and skin [12]. Rab27a is required for the delivery of cytotoxic granules and melanosomes to the plasma membrane in cytotoxic lymphocytes and melanocytes, respectively. In T cells, specific activation leads to partial colocalization of Rab27a/Munc13-4 containing vesicles with cytotoxic granules at the immunological synapse [11]. This indicates that lytic granules and important components of the exocytic machinery are separated into different vesicular compartments in resting lymphocytes and fuse at the time of T cell activation. The place and role of STX11 in this process remains to be characterized.

Analysis performed in this study demonstrates unusual regulation of STX11 in that its expression in human NK cells is strictly dependent on the functional proteasome-mediated protein degradation pathway. We also show that in resting NK cells STX11 is expressed in a subcellular compartment, which is distinct from cytotoxic granules and Rab27a-containing vesicles in resting NKL cells but colocalizes with these structures at the immunological synapse of activated NK cells. Comparative analysis of STX11-expressing and STX11-deficient $CD8^+$ activated T cells suggested that STX11 facilitates fusion of Rab27a/Munc13-4-expressing vesicles with cytotoxic granules. These data reveal a previously unknown level of spatial/temporal complexity in the regulation of exocytosis in cytotoxic lymphocytes, which may be important for strict control over release of cytotoxic granules from NK cells.

Materials and methods

Constructs

The full-length human *STX11* cDNA (Mammalian Gene Collection, clone 34537) was subcloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) *via* 5′ *Kpn*I and 3′ *Not*I cloning sites and the resulting plasmid was sequenced using the following primers: 5′-GGCTCCGTAAAGC and 5′-GCGACACCAACTCC. The STX11 cDNA fragment was ligated with linearized pTriEx5 vector (EMD Chemicals, Novagen. San Diego, CA, USA) at the *Kpn*I/*Not*I sites and modified by PCR to reduce the PolyA tail using the following mutagenic primers: 5′-GTACCAGGCAAAATGAAAGACCGGC-TAGCAGAACTTCTGG (forward) and 5′-GCCCGGAGCTCCTGCGGC-CGCTCTTGAGGCAGGGACAGCAGAAGC (reverse). The sequence of the modified fragment was confirmed with the following sequencing primers: 5′- CGTGCTGGTTATTGTGCTGTCTC and 5′-GCGACACCAACTCCATCG CCAAG. The fragment was inserted upstream of the phrGFP ORF in the phrGFP-IIC expression vector (Stratagene, La Jolla, CA, USA) to generate *STX11*-GFP C-terminal fusion. The pCMV-3XFLAG1a plasmid (Stratagene) was used for FLAG-tagged STX11 expression. The pcDNA3.1-*STX11* plasmid was modified by PCR to introduce *EcoRV* restriction site upstream of *STX11* ORF using the following primers: 5′-GCTGCAGGAATTCGATATC-GACGACAAGGTACC (forward) and 5′-ATCTAGAGGGCCCTATTCTATAGTGT-CACC (reverse). A fragment excised from the modified product with *EcoRV* and *Xho*I was cloned into the pCMV-3XFLAG1a vector. The DNA sequencing was performed using the following primers: 5′-GGCGTGTACG-GTGGGAGGTC and 5′-CGAGAACTTGCTGGCCGACGTG.

Antibodies

Rabbit affinity-purified polyclonal STX11-specific antibodies were generated by Bethyl Laboratories, Inc. (Montgomery, TX, USA) against a synthetic peptide corresponding to the first 15 amino acids of the protein. The following primary antibodies were used for immunostaining: the mouse anti-human LAMP1 monoclonal antibody clone E-5, rabbit polyclonal anti-human CD-M6PR antibody H-251 and rabbit polyclonal anti-human Rab27a antibody H-60 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal anti-FLAG antibody clone M2 from Sigma Aldrich (St Louis, MO, USA); mouse monoclonal anti-human perforin antibody clone δG9 from BD Biosciences (San Jose, CA, USA) and clone Pf-344 from Mabtech AB (Nacka Strand, Sweden). Secondary Alexa Fluor 488-conjugated goat anti-mouse IgG1, Alexa Fluor 568-conjugated goat anti-mouse IgG2a and IgG2b, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG antibodies were all from Invitrogen. The β-actin-specific mouse monoclonal antibody was from Applied Biosystems, Inc.

Cell lines and primary cell cultures

The MON-B1 lymphoblastoid cell line (LCL) was established from peripheral blood mononuclear cells of a healthy blood donor by infection with Epstein-Barr virus. The AA-B1 LCL was generated from an FHL patient with a pre-mature stop codon mutation in the *STX11* gene. The LCLs and major histocompatibility complex (MHC) class I-deficient K562 cell line were maintained in RPMI 1640 medium with 10% foetal calf serum (FCS) and standard supplements [13]. HeLa cells, neuroblastoma cell lines SK-N-BE, SH-SY5Y and SHEP-1 were maintained in IMDM medium with 5% FCS. NKL, NK-92 and KHYG-1 [14] cells were maintained in RPMI 1640 medium with 10% FCS and 10 U/ml of recombinant human IL-2.

Allo-specific CTL lines were generated by consecutive *in vitro* restimulations of peripheral blood lymphocytes with irradiated MON-B1 cells as previously described and propagated in the presence of 10 U/ml of human IL-2 [15]. Primary NK cells and T cells were purified from blood of healthy

donors using negative selection with immunomagnetic beads from Miltenyi Biotec (Auburn, CA, USA) according to the manufacturer's procedure.

Generation of transfectants

HeLa and neuroblastoma cells (SK-N-BE, SH-SY5Y and SHEP-1) were transfected using Lipofectamine 2000 (Invitrogen). NK cell lines were transfected using the nucleofection Amaxa technology (Lonza, Walkersville, MD, USA) using the Cell Line Nucleofector Kit R and X-001 program. AA-B1 cells were transfected using a Human B cell Nucleofector Kit and program U-15. All transfectants were cultured in the presence of 1000 μg/ml of G418 (Cellgro®, Mediatech, Manassas, VA, USA).

Treatment with protease inhibitors and immunoblotting

The following protease inhibitors or inhibitors of lysosomal acidification were used at the indicated final concentrations: lactacystin (10 μM), MG132 (50 μM), MG262 (10 μM), chloroquine (25 μM), ammonium chloride (100 μM), Z-VAD-fmk (50 μM) (all from Sigma Aldrich), AAF-cmk (5 μM) (Santa Cruz Biotechnology). The P1869 cocktail of protease inhibitors containing aprotinin, bestatin, leupeptin, E-64 and pepstatin A from Sigma Aldrich was used at a dilution of 1:1000. Cells were cultured with protease inhibitors overnight, unless indicated otherwise. Total cell lysates were separated by SDS-PAGE using pre-cast 10% gels and a Criterion gel running unit (Biorad, Hercules, CA, USA) and immunoblotting was performed according to standard procedures.

Intracellular staining and FACS analysis

CTLs were activated with MON-B1 cells at a responder:stimulator ratio of 3:1 for 3 hrs, stained with anti-CD8-PerCP followed by intracellular staining with anti-IFN-γ-APC conjugated antibodies (BD). For degranulation experiments CTLs were activated for 2 hrs on surface immobilized CD3-specific antibodies, stained with CD8-specific antibodies and then permeabilized and stained with anti-perforin-APC conjugated antibodies. Cells were analysed by flow cytometry. The data were acquired and analysed on a FACS analyser using CELLQUEST software (BD).

Cytotoxicity assay

Cytotoxic activity of NK cells and CTLs was measured in ^{51}Cr-release assays performed as described [16,17] at the indicated effector:target ratios. To assess the kinetics of ^{51}Cr-release, microtiter plates were spun-down for 2 min at 800 × *g* after addition of effect and target cells and cytotoxicity was terminated using ethylenediaminetetraacetic acid (2 mM). Where indicated, assays were performed in the presence or absence of 100 nM concanamycin A (CMA) (Sigma Aldrich).

Real-time PCR

Reverse transcriptase reaction was performed with total cell lysates using a TaqMan® cells-to-C_T kit and followed by real-time PCR using Applied Biosystems TaqMan® Master Mix and the TaqMan Gene Expression Assays. Two specific primers were designed to amplify the human *STX11* sequence (GeneBank accession number: NM_003764) (5′-GACTACAACCAGGCCGAGATG-3′ (forward primer) and 5′-CGCTGGATGCGGATCTTG-3′ (reverse primer), along with a fluorogenic probe (5′-FAM-AAGCAGCGCGACAACT-NFQ-3′) for detection of the PCR product using Custom TaqMan® Assay Design Tool (Applied Biosystems). *STXBP2* mRNA expression was measured using a pre-designed TaqMan Gene Expression Assay (Hs01100638_m1). All samples were analysed in triplicates using a real-time PCR Applied Biosystems 7300 instrument. The *STX11* and *STXBP2* transcript levels were determined by relative quantitative PCR (qPCR) using comparative C_T values and normalized using the average values of glyceraldehydes-3-phosphate dehydrogenase-specific signals in the same samples.

Conjugate formation

K562 cells (0.5×10^6) were mixed with non-transfected or transfected NKL cells at an effector:target ratio of 1:1. Similar conditions were used for conjugate formation between allo-specific CTLs and Mon-B1 cells. Cells were spun down, incubated in a pellet for 20 min at 37°C and plated onto poly-L-Lysine coated glass slides for other 20 min of incubation at 37°C. Slides were fixed, washed and used for immunostaining.

Immunofluorescence microscopy

HeLa and SK-N-BE adherent cells were incubated overnight on poly-D-Lysine (Sigma Aldrich) coated cover slips. NK or T cells (5×10^4 cells) were placed on cytoslides using a Shandon Cytospin 4 device (Thermo Scientific, Waltham, MA, USA). Conjugates of NKL cell or CTLs with their targets were formed as described earlier. Cells were fixed for 8 min with 3.7% (w/v) paraformaldehyde solution, pH 7.4 at room temperature, washed three times in PBS and blocked for 30 min with 5% human serum in permeabilization buffer (PBS, 0.2% NP40, 1% BSA). After incubation with primary antibodies for 45–60 min, washing and incubation with secondary antibodies for 25–30 min at room temperature, slides were mounted in FluorSave antifade Reagent (EMD Chemicals, Calbiochem) with 2 μg/ml DAPI (Roche, Basel, Switzerland).

Confocal microscopy was performed using a Zeiss LSM510-Meta microscope (Zeiss, Dublin, CA, USA). Images were acquired with a 63×/1.40 Planapo oil DIC objective, HBO 100 W illuminator and Fluorarc power supply for fluorescence. Axiovision software was used on the microscope workstation and LSM Image Browser was applied on the computer desktop to process images. Confocal images were captured in 12 bit scan mode with 1.61 μs pixel time in scan speed 9. Image sets to be compared were acquired in the same acquisition settings and were processed using Volocity 5.0 (Improvision, Inc.) or LSM510 software.

The extent of signal colocalization at the immunological synapse was estimated by computation of the Pearson correlation coefficient (Pierson's *r*) for at least 10 individual conjugated cells. The region of interest was selected following the boundaries of perforin labelling present at the cell–cell contact for each conjugate. Volocity 5.0 software was used for statistical analysis.

Results

Several mechanisms control STX11 expression in a cell-type-specific manner

Immunostaining and confocal microscopy were previously used to visualize STX11 in monocytes, neutrophils, T and NK cells [4,18–20]. However, the subcellular localization of STX11 has never been investigated in relation to the perforin- or Rab27a/Munc13-4-containing compartments in cytotoxic lymphocytes. Furthermore, the previous studies utilized the commercially available STX11-specific monoclonal antibody clone 32 (BD Biosciences). Extensive analysis of several independent batches of clone 32 failed to generate evidence for recognition of human STX11 by this antibody in immunoblotting or immunocytochemistry (data not shown). In contrast, our custom made polyclonal rabbit antibodies revealed a protein band of the predicted molecular weight of STX11, which was absent in lysates of cells derived from patients carrying a premature stop-codon mutation in the *STX11* gene, such as lymphoblastoid AA-B1 cells (Figs 1 and 2). However, strong reactivity with several other proteins of lower electrophoretic mobility (data not shown) precluded the use of these antibodies in cell imaging. Therefore, we generated a number of plasmids encoding either the wild-type *STX11* or its recombinant variant containing the triple FLAG-tag sequence (3FLAG) upstream of the first methionine of STX11. Increased levels of the protein were detected at 24–48 hrs after transfection of HeLa or NKL cells with the wild-type STX11-encoding plasmid (data not shown). However, no increase over the endogenous levels of STX11 expression was observed at 72 hrs post-transfection. Similarly, overexpression of STX11 protein was not detected in NK-92, NKL or HeLa cells stably transfected with pcDNA3-*STX11* (Fig. 1A). Sublines of NK-92 or NKL cells generated by single cell cloning after several independent transfection procedures exhibited the same pattern of ectopic STX11 expression. The levels of ectopic STX11 in AA-B1 transfectants were comparable to endogenous levels of the protein observed in LCLs generated from healthy individuals. In contrast, high levels of STX11 as well as 3FLAG-STX11 were observed in transiently as well as stably transfected SK-N-BE neuroblastoma cells (Fig. 1 and data not shown). Notably, expression of 3FAG-STX11 could not be assessed with the polyclonal antibodies to STX11 because the position of the recombinant protein on SDS-gels overlapped with another protein band, which generated a very strong unspecific signal (data not shown). Collectively, these results indicated that expression of STX11 is tightly controlled in a cell-type-specific manner.

To analyse the mechanisms of this phenomenon, we used real-time PCR to assess the levels of *STX11* mRNA in wild-type AA-B1, SK-N-BE and NKL cells along with their counterparts stably transfected with the pcDNA3, pcDNA3-STX11, pCMV-3FLAG or pCMV-3FLAG-STX11 vectors. In agreement with the observed overexpression of the protein, increased levels of *STX11* mRNA were detected in SK-N-BE cells transfected with STX11-encoding vectors (Fig. 1B). Expression of *STX11* mRNA was increased by more than sixfold also in pcDNA3-*STX11* transfected AA-B1 cells, which did not, however, overexpress STX11 protein above the levels observed in other LCLs established from B-lymphocytes of

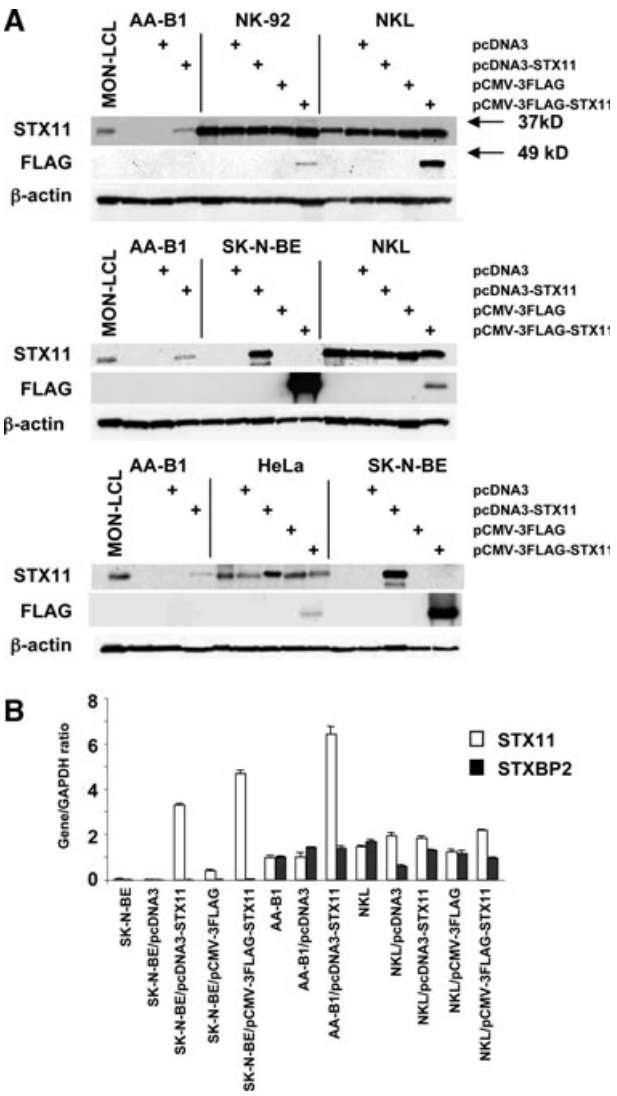

Fig. 1 Endogenous and ectopic expression of STX11 in human cell lines of different origin. (**A**) AA-B1, HeLa, NKL, NK-92 and SK-N-BE cells were transfected with the indicated control vectors or plasmids encoding the wild-type or 3FLAG-tagged STX11. Transfected cells were grown in relevant selection media for at least 2 months before analysis by immunoblotting with the indicated specific antibodies as described in Materials and Methods. Samples were analysed in overlapping sets loaded on the same gel to allow proper comparison of relative expression levels of the wild-type or FLAG-tagged STX11. Total cell lysate of MON-B1 LCL was used as a positive control for each blot. (**B**) Real-time PCR-based quantification of *STX11* and *STXBP2* mRNA expression in the indicated cell lines was performed as described in Materials and Methods. The levels of expression are shown relative to GAPDH expression determined and expressed as 1 for each individual sample. The mean ± S.D. of triplicates.

healthy blood donors, as illustrated by MON-B1 LCL in Figure 1A. Unexpectedly, NKL sublines transfected with STX11 encoding plasmids did not express higher STX11 mRNA levels as compared to control or vector transfected cells. *STXBP2* mRNA was expressed at similar levels in all tested sublines of AA-B1 and NKL cells but was not detectable in SK-N-BE cells (Fig. 1B). These results indicate that expression of *STX11* mRNA in NK cells is controlled through mechanisms, which do not operate in other cell types.

Proteasome activity is required for STX11 expression in NK cells

Several intracellular proteolytic pathways execute post-translational control of protein expression. Incubation of pCMV-3FLAG-STX11-transfected NKL cells with the proteasome inhibitor lactacystin induced accumulation of poly-ubiquitinylated protein species, disappearance of the monomeric form of ubiquitin and, surprisingly, accumulation of LAMP1 (Fig. 2), a lysosomal marker known to be associated with cytotoxic granules [21]. Inhibition of the lysosomal pathway of protein degradation by chloroquine affected neither mono-ubiquitin nor poly-ubiquitinylated protein expression while causing, as anticipated, strong accumulation of LAMP1. Unexpectedly, lactacystin caused nearly complete loss of expression of both the endogenous and ectopic FLAG-tagged forms of STX11. STX11 expression was only slightly increased or unchanged in the presence of chloroquine (Fig. 2A and data not shown).

Enzymatic activity of the proteasome has been shown to positively regulate several promoters [22–24]. However, *STX11* mRNA levels in lactacystin-treated NKL cells were comparable to or exceeded that detected in untreated controls (data not shown). Thus, we investigated whether blockade of other protein degradation pathways can rescue STX11 expression in lactacystin-treated cells. Neither lysosome nor tripeptidyl peptidase II (TPPII) inhibitors affected the steady-state levels of STX11 or prevented the drop in STX11 expression induced by lactacystin in NKL cells. In contrast, incubation with the pan-caspase inhibitor Z-VAD caused notable accumulation of STX11 in untreated cells and almost completely rescued STX11 expression in lactacystin-treated cells (Fig. 2B). Other proteasome inhibitors, MG262 and MG132, also induced STX11 down-regulation in NKL cells (Fig. S1 and data not shown). Treatment with lactacystin induced the same effect in NK92 and KHYG-1 cells (Fig. 2C) as well as primary NK cells purified from the peripheral blood of a healthy blood donor (Fig. 2D). Compared to NK cells, the effect of lactacystin on STX11

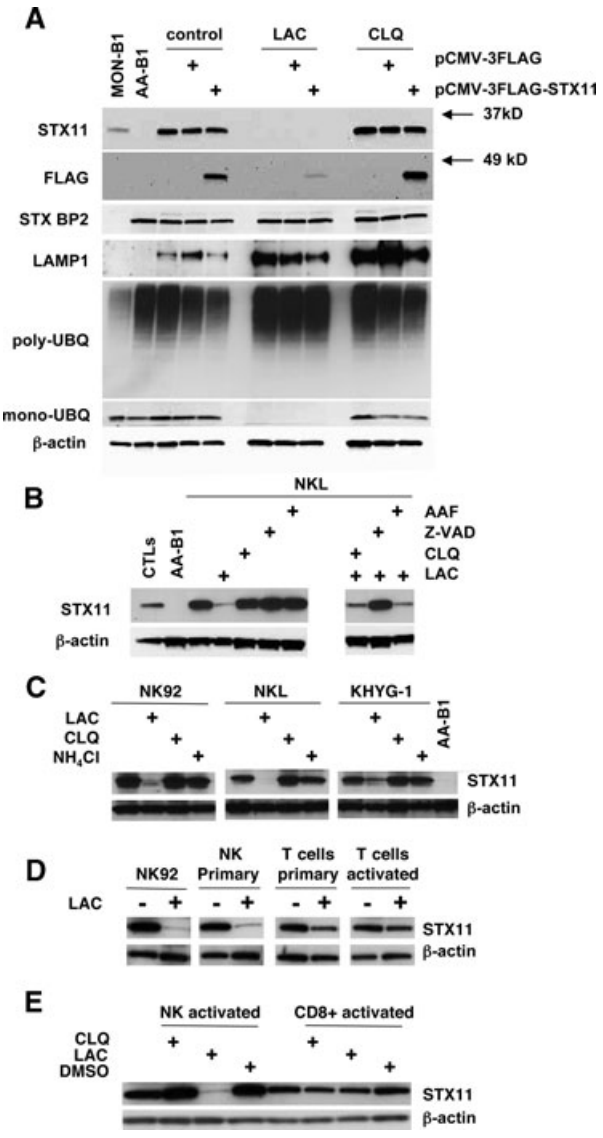

Fig. 2 Expression of endogenous and ectopic STX11 in NK cells requires proteasome-dependent protein degradation. Total cell lysates of untreated (control) and inhibitor treated cells were analysed by immunoblotting with antibodies specific to the indicated proteins. (**A**) Control NKL cells, along with NKL cells transfected with either empty or STX11-encoding pCMV-3FLAG plasmid, were cultured overnight in the presence of lactacystin (LAC, 10 μM) or chloroquine (CLQ, 25 μM). Lysates of AA-B1 and MON-B1 cells were used, respectively, as a negative and positive control of STX11 expression. (**B**) NKL cells were cultured overnight in the presence of lactacystin, chloroquine, the pan-caspase inhibitor Z-VAD (50 μM) or TPPII inhibitor AAF-cmk (5 μM). Inhibitors were added either alone or in combinations as indicated in the figure. CTLs from a healthy donor were included as a positive and AA-B1 cells as a negative control. (**C**) Proteasome but not lysosome inhibitors suppress STX11 expression in the indicated human NK lines. (**D**) Human primary NK cells and T cells were purified from the peripheral blood of healthy donors by negative selection with immunomagnetic beads and then incubated in the absence or presence of lactacystin along with NK92 cells and *in vitro* cultured human CTLs. (**E**) Human primary NK cells and $CD8^+$ T cells were purified as described in (**D**). NK cells were activated by coculturing with irradiated MHC class I negative K562 cells. $CD8^+$ cells were activated by culturing with beads conjugated with CD3- and CD8-specific antibodies. Following activation, NK and $CD8^+$ T cells were cultured in the presence of recombinant IL-2 for 7 days and treated with the indicated protease inhibitors or solvent alone (DMSO) as described in (**A**).

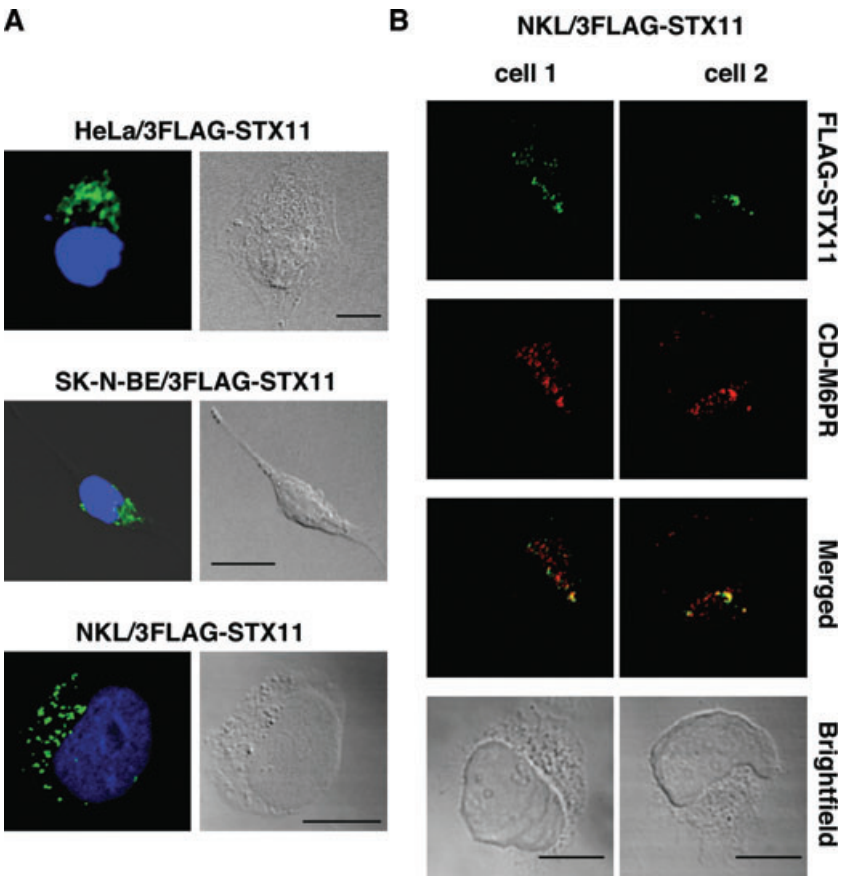

Fig. 3 STX11 resides in a subset of CD-M6PR-expressing vesicles in NKL cells. The indicated cell lines were stably transfected with the pCMV-3FLAG-STX11 plasmid and subcellular localization of STX11 was identified by staining with FLAG-specific antibodies alone (**A**) or in combination with CD-M6PR-specific antibodies (**B**) and confocal microscopy. The scale bars correspond to 5 μm. Analysis of 30 randomly selected individual cells generated an overlap coefficient of 0.74 ± 0.1 for CD-M6PR- and STX11-specific signals.

expression was much less pronounced in purified primary T cells and barely detectable in activated CTLs (Figs 2D and S1). Furthermore, treatment with lactacystin caused strong STX11 down-regulation in purified *in vitro* activated NK cells but not in purified *in vitro* activated $CD8^+$ T cells from the same blood donor (Fig. 2E) Thus, the proteasome mediates its positive effect on STX11 expression in NK cells independently of STXBP2 through interference with STX11 degradation by caspases. Furthermore, this mechanism of control over STX11 expressions appears to be more actively engaged in NK cells as compared to T cells.

STX11 is expressed in intracellular structures largely overlapping with the CD-M6PR-expressing vesicular compartment in NK cells

Having analysed regulation of STX11 expression in several cell types, we next investigated the subcellular localization of the protein in NK cells. Three variants of STX11 encoding plasmids were produced for this study. A variant of STX11 carrying the c-Myc-tag at the amino terminus, failed to be expressed in NKL or HeLa cells (data not shown). A GFP-fusion of STX11 exhibited an unusual behaviour upon expression in HeLa cells. The fusion protein was initially detected in discrete uniformly distributed cytoplasmic patches, but then accumulated in the perinuclear region where the GFP signal gradually decreased until its complete disappearance between 72 and 96 hrs post-transfection (Fig. S2). In contrast, the 3FLAG-tagged STX11 was detected in different cell types and subjected to the same type of proteasome-dependent regulation as the wild-type protein (Figs 1A and 2A). Consistent with the lack of STX11 overexpression observed by Western blotting, immunostaining and fluorescence microscopy revealed FLAG-specific staining only in 5–10% of pCMV-3FLAG-STX11-transfected NKL and 10–15% of HeLa cells but was detected in the majority of transfected neuroblastoma cells. In all cases, FLAG-STX11 staining exhibited a patchy distribution indicating association with cytoplasmic vesicular structures (Fig. 3A). Costaining of 3FLAG-STX11-expressing NKL cells with the relevant antibodies revealed a significant extent of overlap between STX11 and CD-M6PR, a well-characterized marker of late endosomes and the trans-Golgi network (Fig. 3B), consistent with previously demonstrated colocalization of these proteins in HeLa cells [10]. Subcellular fractionation of NK cell lysates identified STX11 in the membrane fraction (data not shown), also consistent with the vesicular nature of the STX11-expressing compartment.

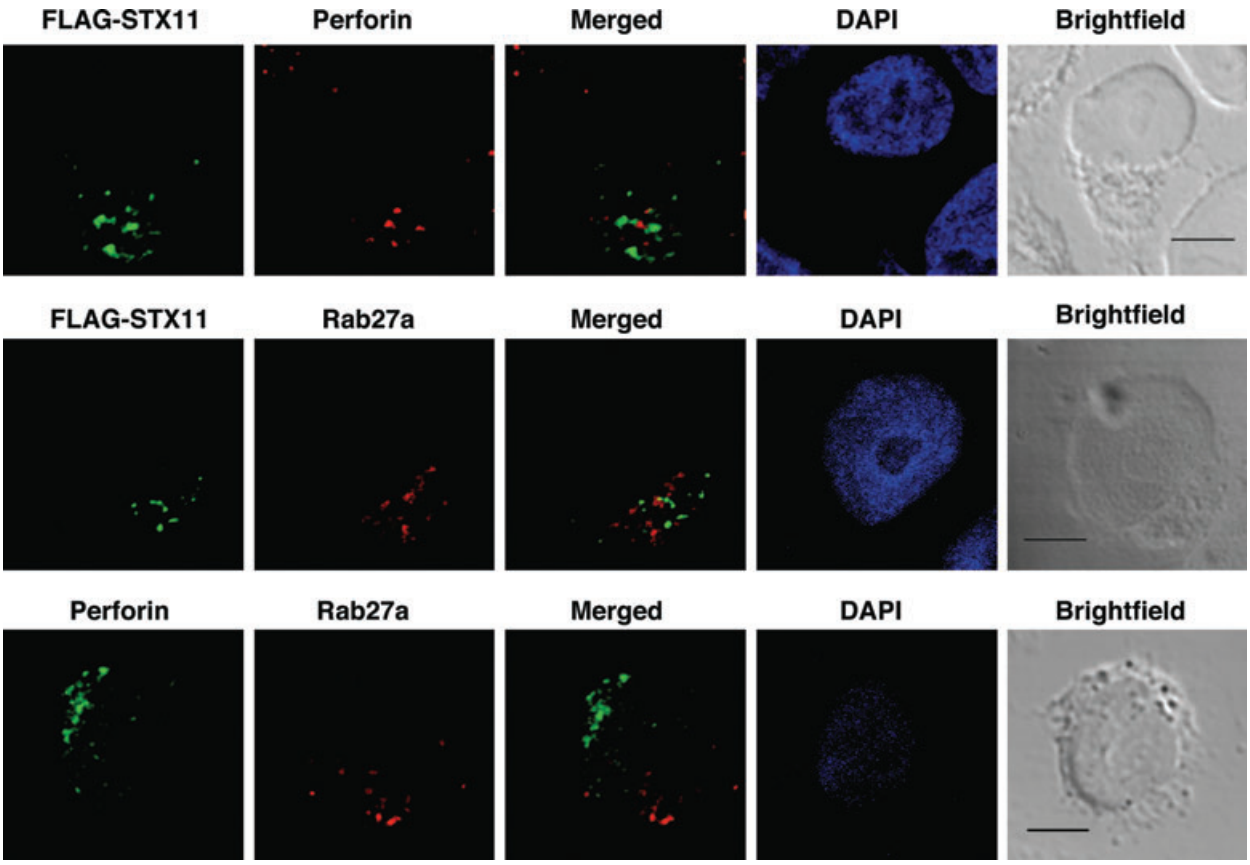

Fig. 4 STX11, perforin and Rab27a localize to different vesicular compartments in resting NKL cells. Confocal microscopy of resting NKL cells stably transfected with pCMV-3FLAG-STX11 was performed after immunostaining with FLAG-, perforin- or Rab27a-specific antibodies. The panels show data representative of three to five independent experiments. The scale bars correspond to 5 μm. Analysis of 30 individual cells generated the following values of overlap coefficient for the indicated pairs of signals: FLAG/perforin-10 ± 5; FLAG/Rab27a-7 ± 2.5; perforin/Rab27a-6 ± 5.

To establish the relationship between STX11-containing structures, Rab27a-expressing vesicles and cytotoxic granules we performed immunostaining of non-activated 3FLAG-STX11-transfected NKL cells with FLAG-, perforin- and Rab27a-specific antibodies in different combinations. Analysis of images obtained by confocal microscopy revealed localization of these proteins in three clearly distinct non-overlapping compartments (Fig. 4).

STX11, Rab27a and perforin colocalize at the immunological synapse in activated NKL cells

Specific activation has been reported to induce colocalization of Rab27a/Munc13-4 expressing vesicles and cytotoxic granules in T cells [11]. After activation of NKL cells by MHC class I-deficient K562 target cells, the three types of intracellular structures defined by expression of STX11, Rab27a or perforin accumulated at the contact area between the effector and target cells (Fig. 5). Confocal microscopy confirmed partial colocalization of STX11 with Rab27a, STX11 with perforin as well as perforin with Rab27a at the area of immunological synapse in activated NKL cells.

Activated STX11-negative T cells exhibit no apparent deficiency of their cytotoxic potential

NK cells from STX11 deficient individuals exhibit impaired degranulation but regain their cytotoxic capacity after a short period of culture in IL-2-containing medium [25]. We next set out to investigate the role of STX11 in CTL-mediated cytotoxicity because degranulation of STX11-deficient CTLs was analysed only in response to polyclonal activation and their specific cytotoxic activity has not been assessed directly [4,25]. We generated allo-specific CTLs from a perforin-deficient FHL (patient 2), two patients carrying homozygous mutations in *STX11* gene, designated as patients 1 and 3, the mother of FHL patient 3 and a healthy unrelated individual. Cultures derived from the healthy blood donor, two independently established cultures from patient 1 and

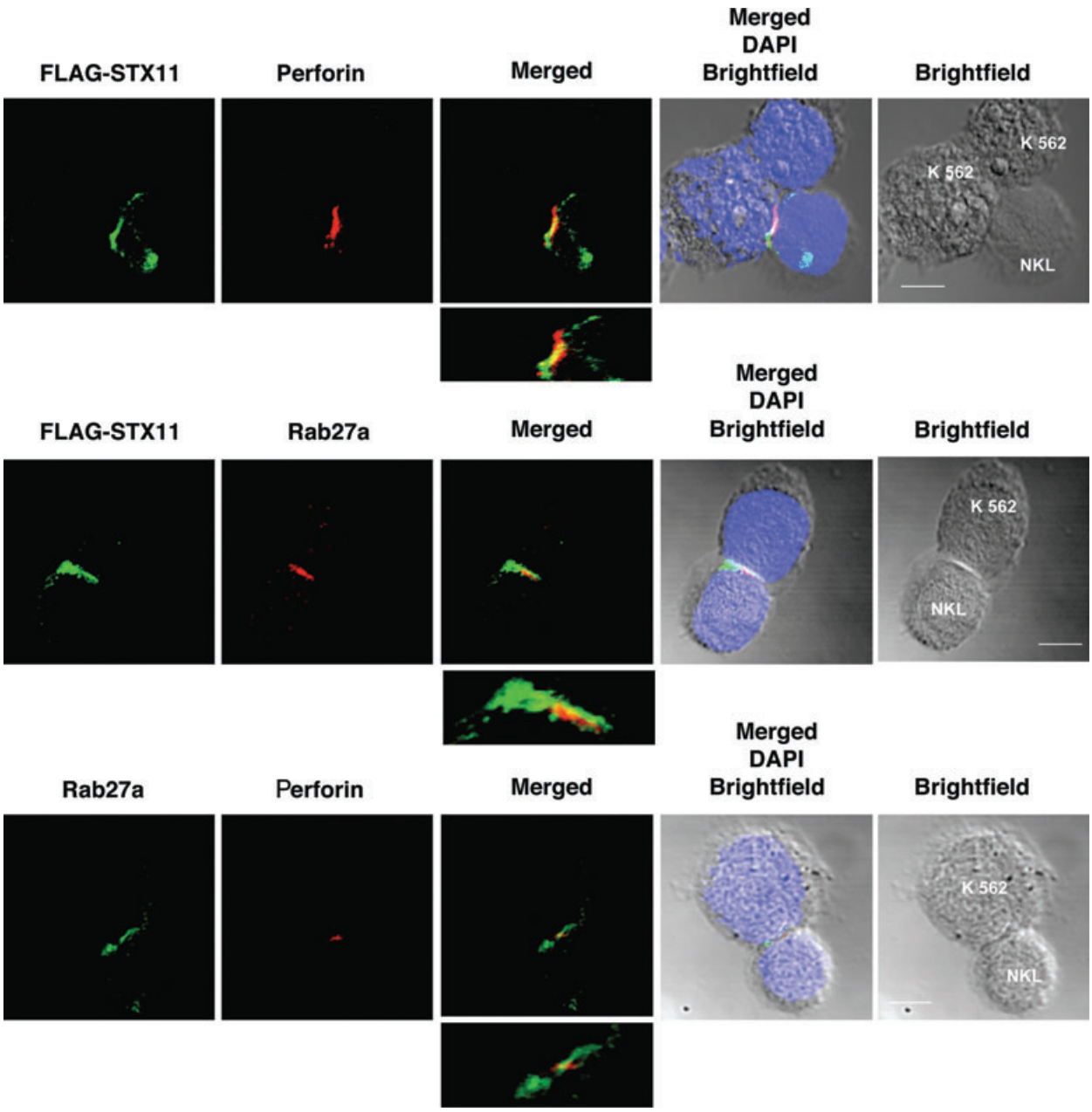

Fig. 5 Activation of NKL cells induces partial colocalization of STX11, perforin and Rab27a at the NK cell/target cell contact area. NKL cells were activated by coculturing with K562 cells for 20 min, fixed and costained with antibodies specific to the indicated proteins and analysed by confocal microscopy. Scale bars, 5 μm. Images are representative of three to five independent experiments. Statistical analysis of 30 individual images produced the following values of Pearson's correlation coefficient: FLAG/perforin, $0.35 > r > 0.67$; FLAG/Rab27a, $0.48 > r > 0.68$; perforin/Rab27a, $0.47 > r > 0.74$.

a culture from patient 2 contained comparable proportions of $CD8^+$ cells (from 40% to 53% of total) as well as $CD8^+$ cell producing IFN-γ in response to specific stimulation (from 60.5% to 68% of $CD8^+$ cells). Cultures established from patient 3 and his mother contained lower but substantial proportions of $CD8^+$ IFN-γ-secreting lymphocytes (9% and 4%, respectively) (Fig. 6A). Cytotoxic activity of these different effectors was then assessed against MON-B1 cells, which were used as stimulators for CTL generation. The assays were performed in the presence or absence of concanamycin A, which disrupts the structure of cytotoxic granules and inhibits perforin maturation [26]. The effector/target ratios in chromium release assays were adjusted to

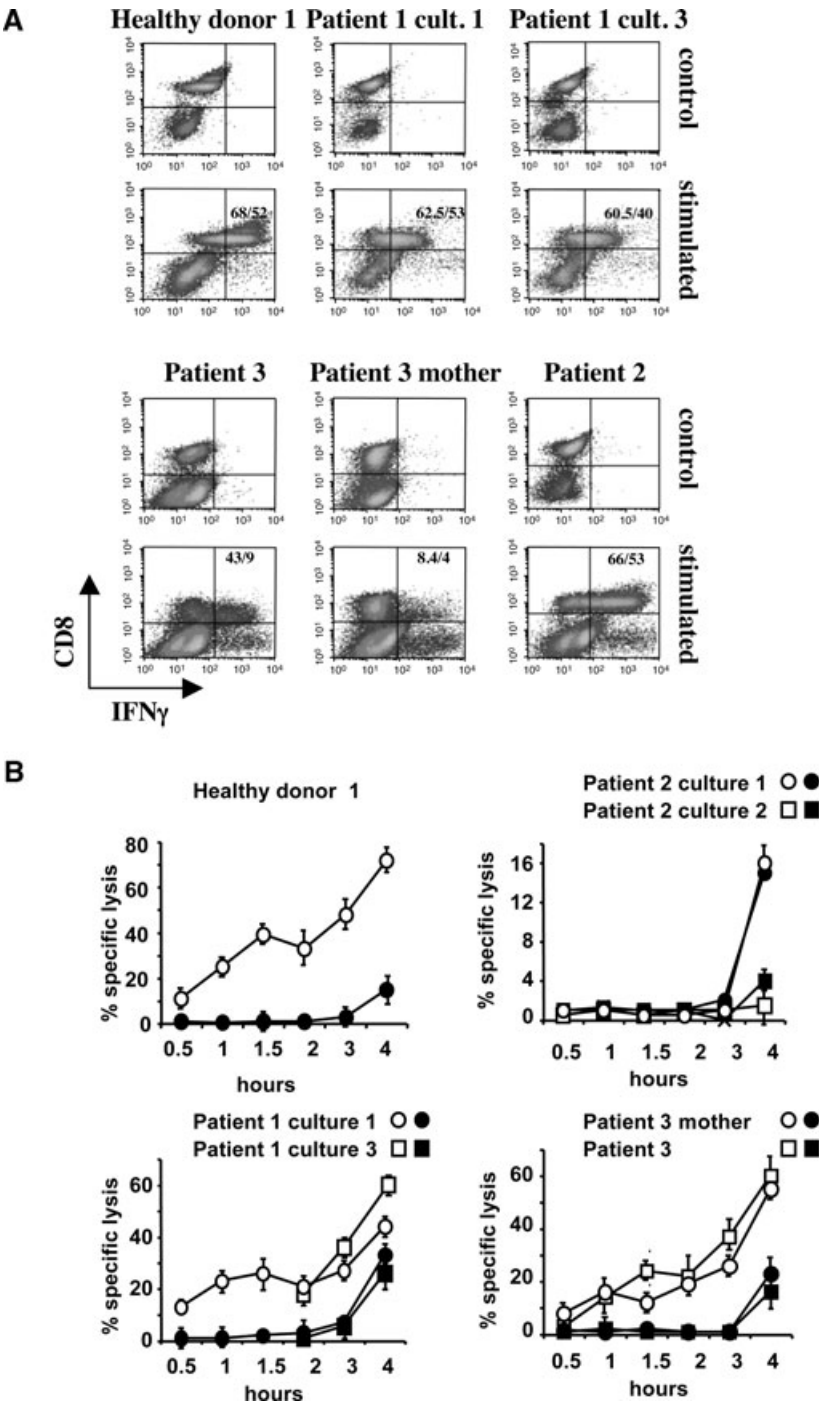

Fig. 6 Composition and cytotoxic activity of allo-specific CTL cultures generated from STX11-deficient or STX11-proficient individuals. (**A**) Cultures of allo-specific T cells were generated from PBLs of a healthy donor, patients 1 and 3 with STX11 deficiency, the mother of patient 3 as well as patient 2 with deficiency in perforin expression. PBLs were subjected to consecutive re-stimulations with the allogeneic MON-B1 LCL and expansion in IL-2 containing medium. IFN-γ production by $CD8^+$ T cells was measured by intracellular immunostaining and FACS analysis before and after stimulation with MON-B1 cells at a responder/stimulator ratio of 5:1. Numbers in the plots represent percentages of $CD8^+$ cells in the cultures and percentages of IFN-γ-producing cells in the $CD8^+$ cell populations. One representative of three experiments. (**B**) Cytotoxic activity of the indicated CTL cultures was tested in ^{51}Cr-release assays. Effectors were added at ratios generating comparable numbers of $CD8^+$ T cells producing IFN-γ in response to stimulation with MON-B1 cells, which served as targets in the assay. The kinetics of cytotoxicity was analysed as described in Materials and Methods. Filled symbols represent cytotoxicity exhibited by the indicated CTL cultures after CMA-induced disruption of cytotoxic granules and inhibition of perforin activity achieved by pre-incubation with CMA for 2 hrs. The error bars show standard deviations of experimental triplicates. One representative of three experiments.

achieve the same number of IFN-γ-producing $CD8^+$ cells per one target cell for each effector preparation. Specific chromium release could be detected 30 min after the initiation of the assay with control CTLs and its level steadily increased reaching 60–70% of target cells lysis after 4 hrs of incubation (Fig. 6B). CMA treatment completely abrogated lysis of target cells by CTLs of healthy donors at the majority of time points. Low level of CMA-insensitive killing observed at later time points was completely blocked by inhibition of Fas/FasL interactions (data not shown). Two CTL lines independently generated from patient 1 were as

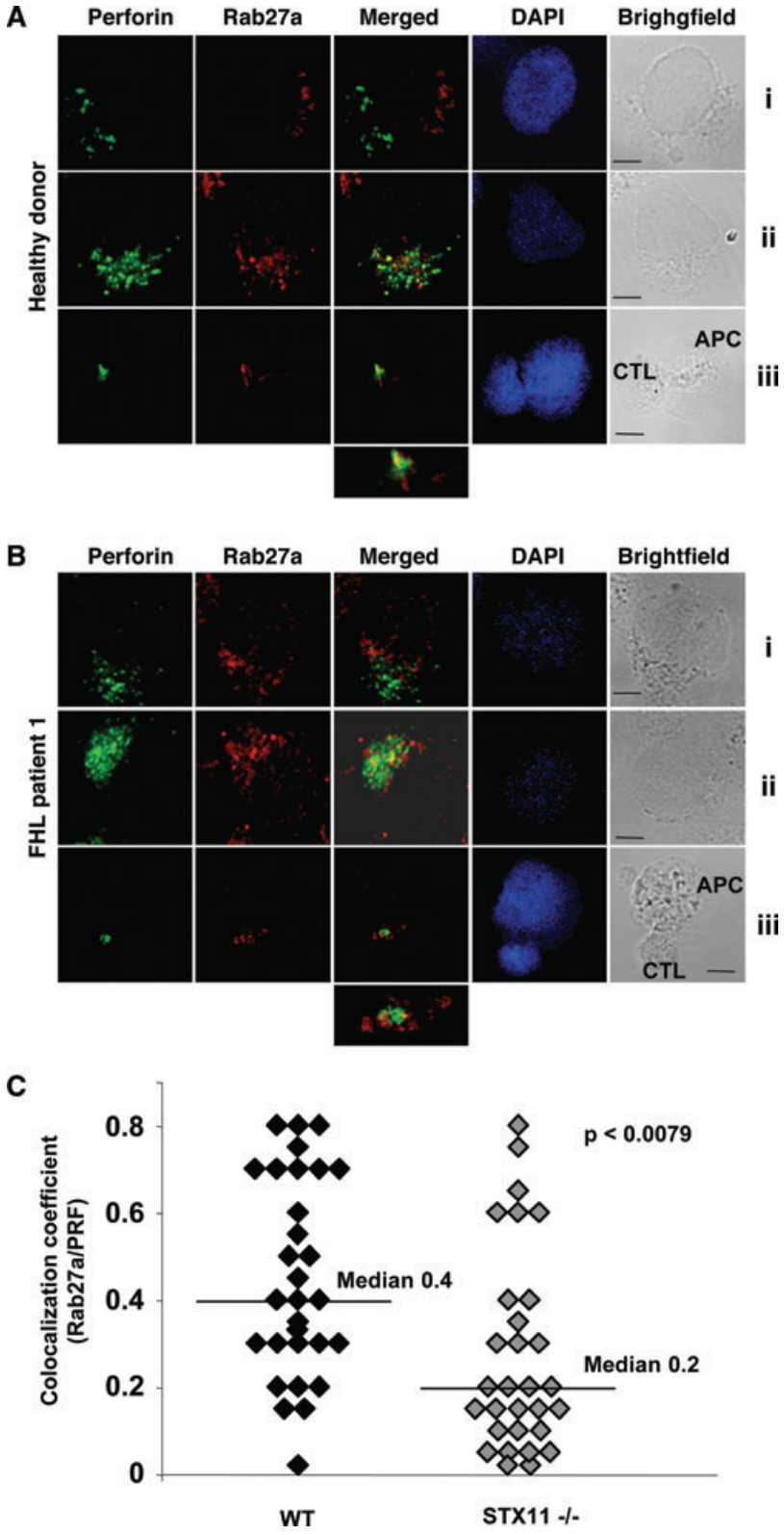

Fig. 7 Spontaneous and activation-induced colocalization of perforin and Rab27a in STX11-deficient and STX11-proficient CTLs. CTLs from a healthy donor (**A**) and FHL patient 1 (**B**) were analysed by immunostaining with perforin- or Rab27a-specific antibodies and confocal microscopy either directly from culture (i, ii) or after 20 min of activation by coincubation with MON-B1 LCLs (iii). Images representative for non-activated CTLs which did not exhibit detectable overlapping of perforin- and Rab27a-specific signal (i), images of cells in which colocalization of the signals was observed without specific CTL activation (ii), and re-localization of perforin and Rab27a into the area of immunological synapse following activation with MON-B1 cells (iii) are shown. (**C**) The extent of colocalization of the two signals was calculated as overlap coefficient assessed for 30 individual cells observed in 10–15 randomly selected fields of view. The statistical analysis of data was performed using Student's two-tailed unequal variance t-test.

efficient in killing target cells as the CTLs obtained from a healthy unrelated donor and were similarly sensitive to CMA treatment at all time points. The kinetics of killing appeared to be slightly delayed, which could be attributed to a lower percentage of IFN-γ-producing cells in the CTL preparations from this individual. In agreement with this notion, no significant differences in the killing capacity, kinetics or CMA sensitivity were observed between CTLs of the FHL patient and his mother (Fig. 6B). Release of cytotoxic granules from STX11-deficient CTLs was also demonstrated by their decreased perforin staining observed after specific stimulation (Fig. S3). Thus, STX11-deficient CTLs do not exhibit any apparent defects of cytotoxic capacity.

STX11-deficient CTLs exhibit decreased spontaneous association of the Rab27a and perforin expressing compartments

Sequence homology of STX11 with other SNARE proteins suggests its participation in the process of membrane fusion [10,27]. Colocalization of STX11 with Rab27a-containing vesicles and cytotoxic granules at the immunological synapse formed by NKL cells indicated that STX11 may promote the fusion of these two compartments. We speculated that STX11 still performs such a function in activated CTLs even though their degranulation is not significantly affected in the absence of the protein. Distribution of Rab27a and perforin was analysed in STX11-negative and STX11-expressing CTLs before and after their specific activation with Mon-B1 cells. In activated STX11-deficient or STX11-expressing CTLs, both compartments accumulated in a similar manner at the CTL:APC contact area demonstrating a significant extent of overlap [Fig. 7A and B(iii)] as was observed for activated NKL cells. In non-activated CTLs, two patterns of Rab27a and perforin distribution were observed. In some cells, Rab27a and perforin were revealed in patchy, vesicle-like structures showing virtually no overlap, whereas in other cells high values of overlap coefficient (up to 0.8) of the two signals were determined through digital analysis of confocal images [Fig. 7A and B(i and ii)]. Analysis of randomly selected individual cells, demonstrated that virtually all T cells in STX11-expressing cultures exhibited some degree of overlap between perforin- and Rab27a-specific signals (median coefficient value 0.4), whereas the overlap coefficient for the two signals did not exceed 0.2 for approximately 50% of STX11-deficient cells (Fig. 7C). Thus, STX11 appears to promote spontaneous fusion of Rab27a vesicles with cytotoxic granules in activated CTLs.

Discussion

In this study we show that in spite of its previously demonstrated role in the process of NK cell cytotoxicity, STX11 is not associated with cytotoxic granules in non-activated NKL cells and resides in a subcellular compartment distinct from Rab27a containing vesicles.

Analysis of STX11 expression performed in this study led to a number of novel observations. First, we showed that stable overexpression of ectopic STX11 cannot be achieved in LCLs, HeLa or NKL cells whereas overexpression of both STX11 mRNA and STX11 protein was apparent in transfected SK-N-BE (Fig. 1), SH-SY5Y and SHEP-1 (data not shown) neuroblastoma cells. Recent data have demonstrated that STX11 expression in cells of haematopoietic origin requires STXBP2 [4,9]. However, this does not seem to be applicable to all cell types, as NB cells do not express STXPB2, as shown by real-time RT-PCR analysis (Fig. 1B) and immunoblotting (data not shown). We cannot exclude that STXBP2 expression restricts the level of STX11 in lymphoblstoid cells, as the relevant transfectants of AA-B1 cells overexpressed STX11 mRNA but expressed the protein at levels comparable to those detected in STX11-proficient LCLs. However, expression of STX11 in NKL cells appears to involve additional mechanisms because transfection with STX11-encoding plasmids failed to induce not only the protein but also STX11 mRNA overexpression in this cell line. Notably, overexpression of GFP was easily achieved in NKL cells after their transfection with a pcDNA3-based GFP-encoding plasmid and IL-15 overexpression was reported in a previously published study [28]. This argues against regulation of ectopic STX11 expression at the level of CMV promoter activity. Thus, the mechanisms regulating STX11 mRNA expression in NKL cells remain unclear and may involve cell-type specific control of STX11 mRNA stability, that would require regulatory elements within the cDNA sequence of the gene. Alternatively, STX11 overexpression may be associated with growth suppression in NKL cells and selection of NKL variants with low activity of the CMV-promotor, which drives expression of the inserted gene in the pCMV-3FLAG plasmid.

Our experiments also showed that STX11 protein is subjected to unusual regulation by several protein degradation pathways. Inhibition of the proteasome resulted in drastic reduction of STX11 expression in human NK cell lines and primary NK cells (Fig. 2). This effect was counteracted by concomitant inhibition of caspases in lactacystin-treated cells, implying the existence of a proteasome-regulated STX11-interacting partner, which serves as a substrate of proteasomal degradation but targets STX11 for degradation by caspases. In agreement with this proposed scenario we observed accumulation of SXT11 in NKL cells treated with pan-caspase inhibitor Z-VAD alone (Fig. 2). Importantly, the loss of STX11 expression could be observed in NKL cells already after 3–4 hrs of exposure to proteasome inhibitors in the absence of any detectable apoptotic changes or caspase activation above the steady state level (data not shown), indicating that the decrease of STX11 expression induced by proteasome inhibitors is not a part of the apoptotic program initiated due to the blockage of proteasome activity. This mechanism of control over STX11 expression seems to be independent of STXBP2, whose expression was not affected by inhibition of proteasomal activity (Fig. 2A).

Both endogenously expressed and ectopic 3FLAG-tagged STX11 were subjected to the same type of proteasome-dependent control, strongly supporting the notion that the recombinant variant behaves physiologically and can be used for visualization of the protein in NKL cells. This became necessary because the

currently available STX11-specific antibodies did not possess sufficient specificity whereas GFP–STX11 fusion protein exhibited an abnormal pattern of intracellular behaviour and could be detected only transiently in cells transfected with the relevant expression vector. In agreement with previously published analysis of subcellular distribution of STX11 in HeLa cells [10], immunostaining and confocal microscopy of NKL cells revealed colocalization of 3FLAG-STX11 with CD-M6PR, a well-characterized marker of late endosomes and trans-Golgi network. Presence of STX11 in the membrane but not in the cytoplasmic fraction of NKL cell lysates was also consistent with association of the protein with a vesicular compartment (data not shown). Unfortunately, direct reliable demonstration of STX11 association with intracellular vesicles in NK cells by electron microscopy is currently hindered by low frequency of NKL cells expressing detectable levels of the FLAG-tagged protein. Unexpectedly, we found that the compartment marked by STX11 expression was clearly distinct from cytotoxic granules as well as Rab27a containing vesicles in resting NKL cells. However, these three structures overlapped in the area of immunological synapse of NKL cells activated by MHC class I-deficient targets. Our data suggest that the cytotoxic machinery, Rab27a GTPase required for granule mobility and fusion, and STX11, which may serve as a regulatory and structural component in membrane fusion, are subjected to strict spatial segregation in NK cells. NK cells can execute their cytotoxic function without pre-exposure to pro-inflammatory factors and, in contrast to T cells, do not require a period of additional differentiation or maturation to become cytotoxic. Thus, spatial segregation of molecules involved in the process of cytotoxic granule release might be required to prevent unwanted NK cell cytotoxicity before their exposure to the necessary combination(s) of activating signals.

It has recently been shown that Munc13-4 and Rab27a are colocalized in CTLs and mast cells in secretory vesicles, which are distinct from cytotoxic granules [11,29]. Strengthened by these published data, our results suggest that STX11 resides in a distinct vesicular compartment participating in the process of CTL and NK cell degranulation. However, a recent publication by Wood et al. suggested that Rab27a and Munc13-4-containing vesicles represent separate entities as these proteins can be independently recruited into the NK/target cell area by triggering different activating receptors on NK cells [30]. Thus, the relation of STX11 to Munc13-4-expressing compartment in *ex vivo* isolated, non-activated NK cells and $CD8^+$ T cells remains to be addressed. This will require development of new antibodies recognizing the native forms of Munc13-4 and STX11 with the level of specificity, which cannot be achieved with the currently available reagents.

Our comparative analysis of STX11-deficient and STX11-proficient T cells demonstrated that in a proportion of activated CTLs, cytotoxic granules and Rab27a expressing vesicles fuse spontaneously. However, the extent of Rab27a and perforin colocalization observed before specific CTL triggering was significantly lower in STX11-deficient cells suggesting that one of the functions of STX11 is to promote the fusion of cytotoxic granules with Rab27a-expressing vesicles. It is clear, nevertheless, that following specific CTL triggering such a fusion event occurs in a STX11-independent manner as colocalization of Rab27a and perforin was observed in virtually all contact areas between antigen presenting cells and STX11-deficient or STX11-expressing CTLs. Reconstitution of degranulation and cytotoxicity in STX11-negative NK cells upon exposure to IL-2 suggests that deficiency of degranulation exists also in resting $CD8^+$ T cells but it is masked in fully activated T cells due to their exposure to TCR-triggering and lymphokines. However, no major impairment in the capacity to express the degranulation marker CD107a at the cell surface in response to anti-CD3-induced activation has been observed in STX11-deficient as well as STXBP2-deficient $CD8^+$ T cells [4], consistent with out data obtained with allo-specific CTLs. These results indicate that functions performed by STX11 in NK cells and CTLs are not fully overlapping. This notion is strongly supported by our results demonstrating that STX11 expression is much more dependent on the activity of the proteasome-mediated protein degradation in NK cells as compared to T cells. These differences in the biochemical regulation, strongly suggest that STX11 is involved in different sets of protein–protein interactions in the two cell types. It remains to be seen whether the fusion of Rab27a-expressing vesicles and cytotoxic granules induced by triggering of NK-activating rectors is more dependent on STX11 in NK cells than that induced by TCR-triggering in T cells. In support of this possibility, we have never observed spontaneous fusion of cytotoxic granules with Rab27a-expressing vesicles in NKL cells or primary NK cells (Fig. 4 and unpublished results). It cannot be excluded that STX11 performs additional functions in the process of CTL and NK cell degranulation and that the STX11-expressing vesicular compartment delivers structural or regulatory components absent in cytotoxic granules of resting lymphocytes but required for their proper release and/or post-exocytic function. Further comparative analysis of STX11 regulation and function in T and NK cells is likely to improve our understanding of the specific role of different lymphocytes subsets in FHL pathogenesis. This, in turn, may lead to the development of new refined approaches of cellular and molecular therapy of this detrimental condition.

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Conflict of interest

The authors confirm that there are no conflicts of interests.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Different proteasome inhibitors suppress STX11 expression in NKL cells.

Fig. S2 Kinetics of STX11-GFP fusion protein expression in transiently transfected HeLa cells.

Fig. S3 Activation-induced cytotoxic granule release from $STX11^-$ and $STX11^+$ CTLs as determined by perforin-specific staining.

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