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REVIEW

SPECIAL ISSUE: CELL BIOLOGY OF THE IMMUNE SYSTEM

Molecular regulation of the plasma membrane-proximal cellular steps involved in NK cell cytolytic function

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ABSTRACT

Natural killer (NK) cells, cytolytic lymphocytes of the innate immune system, play a crucial role in the immune response against infection and cancer. NK cells kill target cells through exocytosis of lytic granules that contain cytotoxic proteins, such as perforin and granzymes. Formation of a functional immune synapse, i.e. the interface between the NK cell and its target cell enhances lysis through accumulation of polymerized F-actin at the NK cell synapse, leading to convergence of lytic granules to the microtubule organizing center (MTOC) and its subsequent polarization along microtubules to deliver the lytic granules to the synapse. In this review, we focus on the molecular mechanisms regulating the cellular processes that occur after the lytic granules are delivered to the cytotoxic synapse. We outline how – once near the synapse – the granules traverse the clearings created by F-actin remodeling to dock, tether and fuse with the plasma membrane in order to secrete their lytic content into the synaptic cleft through exocytosis. Further emphasis is given to the role of Ca^{2+} mobilization during degranulation and, whenever applicable, we compare these mechanisms in NK cells and cytotoxic T lymphocytes (CTLs) as adaptive immune system effectors.

KEY WORDS: Exocytosis, Immune deficiency, Lytic granule, Natural killer cell

Introduction

NK cells are cytotoxic lymphocytes of the innate immune system and represent the first-line immune defense in the elimination of virally infected cells, stressed cells and cancer cells. They detect abnormal cells through the interactions of activating receptors that are expressed on the NK cell surface with their corresponding ligands, whose expression is generally increased on the surface of stressed and infected cells (Cerwenka and Lanier, 2001; Vivier et al., 2012). A large array of activating receptors, such as the family of natural cytotoxicity receptors (NCRs; i.e. NCR3, NCR2 and NCR1, hereafter referred to as NKp30, NKp44 and NKp46, respectively), natural killer group 2D (KLRK1, hereafter referred to as NKG2D), DNAX accessory molecule-1 (CD226, hereafter referred to as DNAM1), natural killer cell receptor 2B4 (CD244, hereafter referred to as 2B4), cluster of differentiation (CD)160 [i.e. Fc fragment of IgG receptor IIIa and b (FCGR3A and B, hereafter referred to as CD16), T-cell surface antigen CD2 (hereafter referred to as CD2), and CD94-natural killer group 2C (KLRC2, also known as NKG2C) are expressed, providing broader ability to detect target cells expressing a diverse set of ligands (Vivier et al., 2012). Upon receptor-ligand binding, signals are transmitted inside NK cells to trigger cytolytic activity (Vivier et al., 2008). NK cells possess specialized lysosomal organelles called lytic granules, which contain cytotoxic molecules (Smyth et al., 2002). The process of releasing the lytic content into the extracellular space through lytic granule exocytosis is called degranulation. The main cytolytic proteins in the lytic granules are perforin (Prf) and granzymes (Smyth et al., 2005; Trapani and Smyth, 2002). Besides, perforin and granzymes, NK cells also express apoptosis-inducing ligands that belong to the tumor necrosis factor (TNF) family, namely the FAS ligand (FASL, officially known as FASLG) and the TNF-related apoptosis-inducing ligand (TRAIL, officially known as TNFSF10) either in a membrane bound form and/or as a part of lytic granules (Smyth et al., 2002). Of the multiple mechanisms within the NK cell arsenal for target cell lysis, release of perforin and granzymes through degranulation is the predominant one (Smyth et al., 2002; Krzewski and Coligan, 2012). Upon release, perforin forms pores in the plasma membrane or in the endosomal membrane and facilitate entry of granzymes into the cytosol of target cells (Smyth et al., 2005). Granzymes are serine proteases that induce apoptosis in a caspase-dependent and -independent manner (Krzewski and Coligan, 2012).

The process of directed secretion of cytotoxic molecules at the interface of NK and target cells through degranulation is a tightly regulated multistep process: it is set in motion by the initial contact between the NK cell and the potential target cell, which is likely to be mediated through tethering receptors, such as CD2, and receptors that belong to the selectin family, such as CD62L (Chen et al., 2005; Orange et al., 2003). Lymphocyte function-associated antigen 1 (ITGAL, hereafter referred to as LFA1) and macrophage receptor 1 (MAC1), receptors expressed on NK cells that belong to the integrin family establish firm adhesion between cells through their interactions with intercellular adhesion molecule 1 (ICAM1) on target cells (Orange, 2008). Activating receptors, such as NCRs, NKG2D and DNAM1, also contribute to NK adhesion through their interactions with their corresponding ligands on target cells; moreover, these receptors – along with LFA1 – transmit activating signals inside the NK cells to initiate the formation of immune synapses (Orange, 2008). The activating signals trigger filamentous actin (F-actin) reorganization, which is the first crucial step required for the commitment to synapse formation (Mace et al., 2014). Accumulation of F-actin at the synapse is a pre-requisite for the clustering of activating receptors. Synergistic signals from the cluster of activating receptors stimulate polarization of cytolytic granules to the synapse (Bryceson et al., 2005). For directed secretion of perforin and granzymes into the synaptic cleft, cytolytic granules that are dispersed throughout the cell have to be congregated near the synapse. This is achieved first by movement of granules along microtubules to converge at the microtubule organizing center (MTOC), a cellular structure where microtubule formation begins and from which microtubules radiate in all directions, followed by polarization of the MTOC together with

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lytic granules to the synapse (Orange, 2008). Once near the synapse, the granules traverse the clearings created by F-actin remodeling to dock, tether and fuse with the plasma membrane to secrete their lytic content into the synaptic cleft through exocytosis (Mace et al., 2014; Orange, 2008). Our understanding of the molecular regulation of the synapse-proximal steps involved in degranulation is mainly derived from investigations into the genetic causes of the immune disorders (see Table 1), particularly familial hemophagocytic lymphohistiocytosis (FHL), which are characterized by impaired cytolytic function of the lymphocytes (Sieni et al., 2014, 2012). Inability to clear infections due to deficient lymphocyte killing leads to hyperinflammation and uncontrolled proliferation of lymphocytes and macrophages in FHL patients. Cytolytic function of T and NK cells is similarly impaired in these patients, indicating common molecular requirements for degranulation in both cells (Chiang et al., 2013).

In this review, we discuss the molecular mechanisms involved in the plasma membrane-proximal steps of lytic granule exocytosis in NK cells. We start by highlighting the importance of the actin-myosin network to initiate granule movement toward the membrane and continue by elucidating the factors involved in docking and tethering of lytic granules. Furthermore, we review the influence of Ca^{2+} mobilization on degranulation and conclude with the granule-membrane fusion steps that are required for lytic content release. Since T and NK cells have common requirements, molecular processes in cytotoxic T lymphocytes (CTLs) are also discussed wherever necessary.

Movement of lytic granules near the immune synapse

Myosin IIa transports granules along the F-actin network

Lytic granules navigate the dense actin meshwork deposited at the synapse and reach the plasma membrane through gaps that are appropriately sized to accommodate lytic granule movement (Rak et al., 2011). Prior to degranulation, lytic granules are highly motile at the cell cortex (Mace et al., 2012) and their movement is dependent upon non-muscle myosin heavy chain IIa (M|YH9, hereafter referred to as myosin IIa) that generates force and movement along actin filaments (Sanborn et al., 2009). Consequently, inhibition or knockdown of myosin IIa blocks lytic granule exocytosis and cytotoxicity of NK cells, but not conjugate formation or MTOC polarization (Andzelm et al., 2007). Similar impairment was observed in NK cells of patients suffering from MYH9-related disorder, who carry mutations in *MYH9* (Sanborn et al., 2011, 2009) (see Table 1). The phenotype was more pronounced in patients carrying mutations in the N-terminal head region and the neighboring sub-fragment 2 of the myosin IIa protein – which is required for F-actin binding and ATP hydrolysis for motor function – and the C-terminal tailpiece that is crucial for binding to cargo, including the site of phosphorylation to regulate myosin IIa-granule binding (Sanborn et al., 2011, 2009). Lytic granules failed to penetrate into F-actin deposited at the immune synapse, indicated by reduced colocalization of perforin (lytic granules) and F-actin at the immune synapse in myosin IIa-deficient cells (Andzelm et al., 2007); as a result, these cells showed significantly reduced degranulation (Sanborn et al., 2009). Total internal reflection fluorescence (TIRF) microscopy analysis demonstrated less velocity, less total displacement and a lower rate of displacement of lytic granules at the immune synapse in NK cells treated with the myosin IIa inhibitor ML-9, indicating a requirement of myosin IIa for traversing the cell cortex to reach the plasma membrane (Sanborn et al., 2009). In NK cells, myosin IIa is constitutively associated with the surface of lytic granules and the interaction is mediated through a constitutively phosphorylated serine residue at position 1943 in the C-terminal tail of myosin IIa (Sanborn et al., 2011). YTS cells (an NK cell line) that express a C-terminal truncation or a form of myosin IIa with a phospho-dead mutation at amino acid 1943 show reduced colocalization of lytic granules and myosin IIa and decreased cytotoxicity (Sanborn et al., 2011). In addition, lytic granules that were isolated from MYH9-related disorder patients carrying the truncation mutation do not adhere to an F-actin-coated surface (Sanborn et al., 2009). These findings demonstrate that myosin IIa constitutively associates with the lytic granule surface and mediates the interaction of the lytic granule with F-actin (Sanborn et al., 2011, 2009) (Fig. 1).

UNC-45A is a chaperone protein that binds to myosin IIa to affect its folding and stability, thereby enabling its efficient binding to actin (Shi and Blobel, 2010). In accordance with this, knockdown of UNC-45A leads to impaired cytotoxicity in human NK cells and the NK cell line YTS (Iizuka et al., 2015). UNC-45A colocalizes with myosin IIa and F-actin at the immune synapse in NK cells and, together with myosin IIa, UNC-45A associates with lytic granules (Iizuka et al., 2015). Similar to knockdown of myosin IIa, loss of UNC-45A does not affect conjugate formation and MTOC polarization, but reduces degranulation in NK cells. However, whereas knockdown of UNC-45A in the YTS cell line decreases the ATP-dependent interaction of myosin IIa with actin (Iizuka et al., 2015), it does not affect expression of myosin IIa or its phosphorylation levels; this indicates that – at least in this NK cell line – UNC-45A is not required for myosin IIa stability or its folding.

Thus, myosin IIa mediates interaction between lytic granules and F-actin in an UNC-45A-dependent fashion at the immunological synapse in NK cells, and powers the movement of lytic cargo along actin filaments in the cell cortex (Fig. 1).

Table 1. Immune disorders or syndromes that were instrumental in the discovery of molecules involved in NK cell lytic degranulation

Immune disorder/syndrome	OMIM code	Disease-causing mutated gene	Function in NK cell cytolytic activity
MYH9-related disorder	160775	*MYH9* (myosin IIa heavy chain	Lytic granule movement along F-actin at the cell cortex
Griscelli syndrome type 2	607624	*Rab27a*	Lytic granule movement along F-actin at the cell cortex, Docking and tethering of lytic granules at the immune synapse
Familial hemophagocytic lymphohistiocytosis, variant 3 (FHL3)	608898	*UNC13D* (Munc13-4)	Lytic granule maturation, Tethering of lytic granules at the immune synapse, Ca^{2+} sensor
FHL4	603552	*STX11* (syntaxin-11)	Fusion of lytic granule with the plasma membrane
FHL5	613101	*STXBP2* (syntaxin-binding protein 2)	Fusion of lytic granule with the plasma membrane

OMIM, Online Mendelian Inheritance in Man database.

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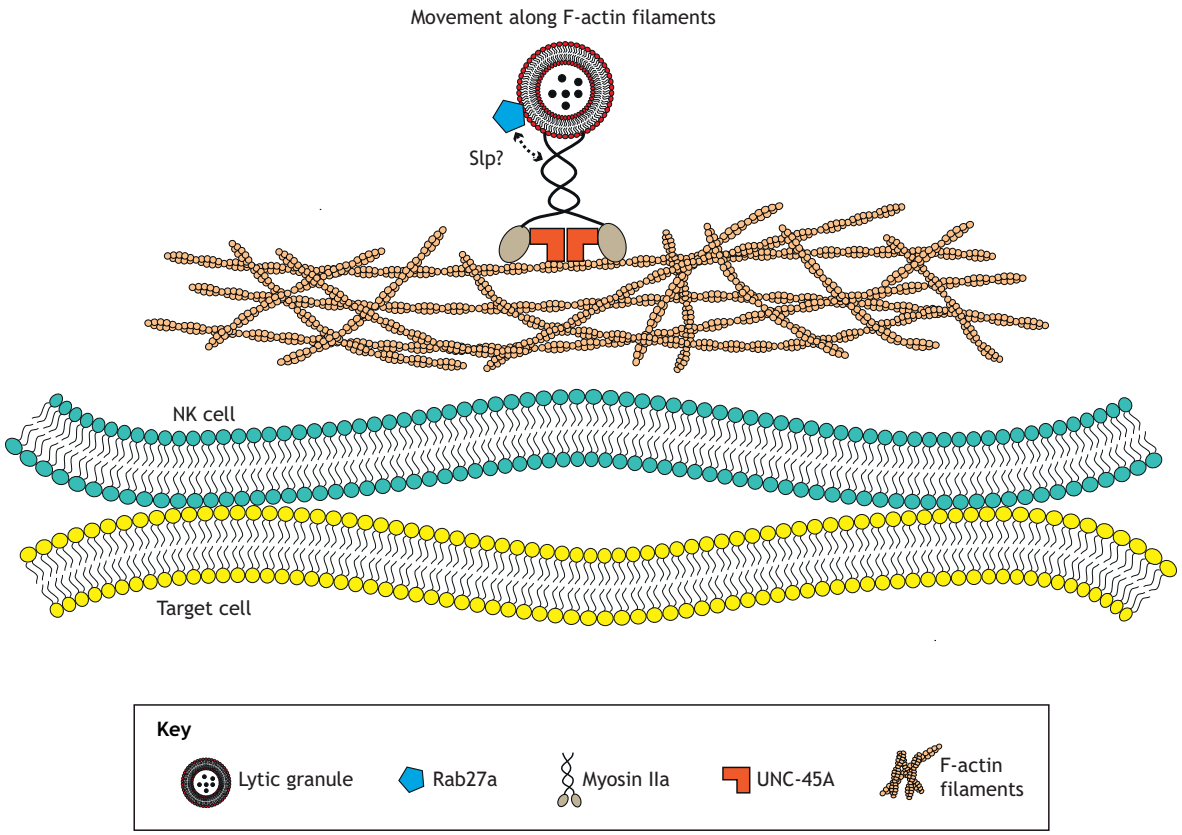

Fig. 1. Movement of lytic granules near the immune synapse. Upon delivery to the immune synapse through MTOC polarization, lytic granules navigate the dense F-actin meshwork and reach the plasma membrane through less-dense actin filaments. Movement of lytic granules near the immune synapse in NK cells is dependent upon lytic granule-associated non-muscle actin motor myosin IIa, which generates force and movement along actin filaments. UNC-45A is a chaperone that binds to myosin IIa, affecting its folding and stability, thereby enabling efficient binding of myosin IIa to actin. The small G-protein Rab27a is also required for movements near the immune synapse. In non-immune cells, Rab27a forms a complex with myosin motor proteins through its effector synaptotagmin-like proteins (Slps); however, in NK cell this possibility has not yet been explored (SLP?).

The role of Rab27 in lytic granule transport

Rab27 belongs to the Rab family of small GTPases, which regulate multiple types of intracellular membrane trafficking and is found in two isoforms, Rab27a and Rab27b, of which Rab27a plays a crucial role in NK cell degranulation (Wood et al., 2009). Like other GTPases, Rab27 switches between a GTP-bound, active state and a GDP-bound, inactive state (Fukuda, 2013). Its role in lymphocytic cytotoxicity was brought to light by the impaired T cell cytotoxicity and cytolytic granule exocytosis observed in Griscelli syndrome type 2 patients (see Table 1), which are characterized by silvery hair attributed to defective melanosome transport in melanocytes and mutations in the *RAB27a* gene (Ménasché et al., 2000). NK cells from Griscelli syndrome type 2 patients also show impaired degranulation and cytotoxicity (Wood et al., 2009). Similarly, T and NK cell cytotoxicity defects were observed in the *ashen* mouse, the corresponding mouse model of Griscelli syndrome type 2, which comprises absent or severely diminished expression of *Rab27a* (Haddad et al., 2001; Stinchcombe et al., 2001). In *ashen* mice, lytic granules were found to accumulate near the plasma membrane of T cells at the site of contact with the target cell, indicating that polarization of MTOC and granules along microtubules toward the synapse was normal (Stinchcombe et al., 2001). However, in contrast to wild-type mice, *ashen* mice granules did not show a tightly focused immune synapse ring. Electron microscopy analysis of conjugates formed by T cells from *ashen* mice or from Griscelli syndrome type 2 patients showed granules lining up near the immune synapse but no docking at the plasma membrane (Stinchcombe et al., 2001; Feldmann et al., 2003) and absence of granule content in the synaptic cleft (Stinchcombe et al., 2001). These findings demonstrate that Rab27a is not required for the polarization of MTOC and lytic granules along microtubules toward the immune synapse but that it is essential for the plasma membrane-proximal movement of granules (Stinchcombe et al., 2001). The role of Rab27a in lytic granule movements near the plasma membrane was further established by the observation that, in resting Rab27a-deficient NKL cells (an NK cell line used to study human NK cells) (Robertson et al., 1996) and in NK cells from *ashen* mice, fewer lytic granules reside near the plasma membrane (Liu et al., 2010). The lytic granules, whose movement is both actin- and microtubule-dependent near the plasma membrane, are also less mobile in inactivated Rab27a-deficient NK cells (Liu et al., 2010). Thus, Rab27a seems to regulate lytic granule movements near the plasma membrane (Fig. 1) not only in activated but in resting NK cells as well. In the following section, we discuss possibilities of how Rab27a exerts its function in the movement of lytic granules.

A role for Rab27a effectors in the NK cell immune synapse-proximal movements?

Rab27a mediates its functions – cytoskeleton-based transport of secretory vesicles and their docking at the plasma membrane – in various secretory cells through effectors that bind to its active form (Fukuda, 2013). In melanocytes, Rab27a binds Slp homolog lacking C2 domains-a (MLPH, hereafter referred to as SlaC2-a) and forms a complex with MYO5A (hereafter referred to as myosin-

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Va) to transport melanosomes along actin filaments (Strom et al., 2002; Wu et al., 2002; Fukuda et al., 2002; Provance et al., 2002). Whether Rab27a forms a complex with myosin IIa in NK cells is not known. The possible effector proteins that can form a link between Rab27a and myosin IIa in NK cells are synaptotagmin-like proteins (Slps). Interestingly, Slp1, Slp2a and Slp3 (officially known as Sytl1, Sytl2 and Sytl3, respectively) are expressed in T and NK cells, and bind to Rab27a (Holt et al., 2008; Ménasché et al., 2008; Kurowska et al., 2012). Slp proteins are also known to bind Rab27a in order to form a complex with motor proteins: in pancreatic β cells, Slp4a (officially known as Sytl4) binds to myosin-Va, and facilitates complex formation between Rab27a and the motor protein to regulate movement of insulin granules along actin (Brozzi et al., 2012). In CTLs, Slp3 binds to the microtubule-based motor protein kinesin-1 to form a complex with Rab27a (Rab27a–Slp3-kinesin-1 complex), which enables polarization of lytic granules along microtubules to the immune synapse (Kurowska et al., 2012). However, complex formation between Rab27a, Slps and myosin IIa, and its functional requirement has not been explored in NK cells. In this regard, investigating actin-based movements of lytic granules near the immune synapse in NK cells from Griscelli syndrome type 2 patients may prove useful. In summary, myosin IIa mediates interaction between lytic granules and F-actin in an UNC-45A-dependent fashion at the immunological synapse in NK cells, and facilitates the movement of lytic cargo along actin filaments. Rab27a is also required for immune synapse-proximal movements of lytic granules. Apart from regulating the movement of lytic granules near the immune synapse, Rab27a is also involved in other processes occurring near the plasma membrane, which are discussed in the following section.

Docking and tethering of lytic granules at the immune synapse

Docking of secretory vesicles to the plasma membrane occurs through the activity of Rab27a and its effectors, which anchor to the plasma membrane through their C2 domains (Fukuda, 2006). Slps, rabphilin 3A (RPH3A) and UNC13D (hereafter referred to as Munc13-4; see below) contain a Rab27a-binding as well as C2 domains; in the case of Slps, the N-terminal Slp-homology domain (SHD) binds Rab27a and the C-terminal tandem C2 domains bind phospholipids (Fukuda, 2002, 2013; Fukuda et al., 2001; Neeft et al., 2005; Shirakawa et al., 2004) (Fig. 2). Slp2a anchors Rab27a-associated melanosomes to the plasma membrane of melanocytes (Kuroda and Fukuda, 2004), whereas Slp4a links Rab27a-bearing vesicles to the plasma membrane in MDCK cells (Gálvez-Santisteban et al., 2012). Slp1 and Slp2a might have a similar role in activated CTLs as they localize to the plasma membrane, and Slp2a focuses at the site of contact in the immunological synapse (Holt et al., 2008). In CTLs, Rab27a stabilizes Slp2a to prevent it from being quickly degraded. Furthermore, Slp2a colocalizes with Rab27a to vesicles, and binding of Slp2a to Rab27a is required for its vesicular localization (Holt et al., 2008; Ménasché et al., 2008). Slp2a and Rab27a colocalize with perforin to the immunological synapse in CTLs, and expression of the Slp2a C2 domain prevents localization of Rab27a to the immunological synapse in CTLs, indicating a requirement of Slp2a in docking Rab27a-bearing vesicles at the immunological synapse. The role of Slp2a in docking and subsequent exocytosis of lytic granules was further reinforced by the significantly reduced degranulation observed in CTLs that only express the SHD of Slp2a, which sequesters Rab27a from full-length Slp2a protein (Ménasché et al., 2008). However, cytotoxicity of CTLs from Slp1-

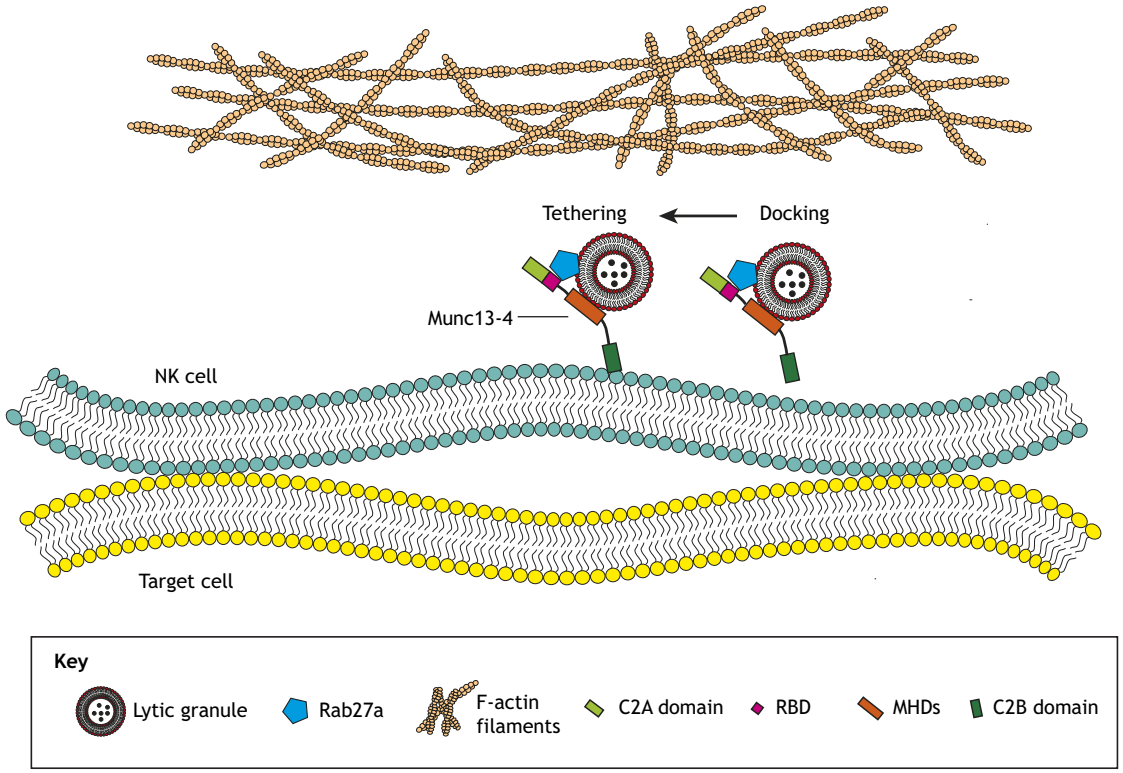

Fig. 2. Docking and tethering of lytic granules at the immune synapse. After navigating the dense F-actin meshwork accumulation at the synapse, lytic granules reach the plasma membrane. Rab27a is essential to localize the granules close to the plasma membrane (docking). The Rab27a effector protein Munc13-4 interacts through its Rab27a-binding domain (RBD) with Rab27a and associates with lytic granules through its Munc-homology domains (MHDs). Munc13-4 mediates the tethering of docked granules to the plasma membrane through its phospholipid-binding C2B domain.

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or Slp2a-knockout mice was found to be unaffected (Holt et al., 2008; Ménasché et al., 2008). Expression of the SHD of Slp2a, which is 56% identical and 73% similar in amino acid sequence compared to that of Slp1 – and, thus, might act as a dominant-negative mutant for both Slps – only partially reduced CTL cytotoxicity (Holt et al., 2008). These findings indicate that there are other Rab27a effectors that can substitute for Slps in docking lytic granules at the immunological synapse of cytotoxic lymphocytes.

Interaction between Rab27a and Munc13-4

In addition to the above, Rab27a binding to Munc13-4 is essential for degranulation of lytic granules in cytotoxic lymphocytes (Elstak et al., 2011). Munc13-4 belongs to the Munc13 family of proteins, which are predominantly expressed in the nervous system. However, it differs from other members of the family in not having the N-terminal diacyglycerol-binding C1 domain and being expressed outside of the nervous system (Neeft et al., 2005). Like other Rab27a effectors, Munc13-4 does contain neither C2 domains in tandem nor a Rab27a-binding SHD but has two separate C2 domains that flank a unique Rab27a-binding domain (RBD) and Munc homology domains (MHDs) (Neeft et al., 2005; Fukuda, 2013) (Figs 2 and 3). Munc13-4 is predominantly expressed in hematopoietic cells (Feldmann et al., 2003), including CTLs and NK cells, and its expression increases with the acquisition of lytic granules and cytolytic maturation (Cichocki et al., 2014), which is in line with its role in the maturation of lytic granules as described earlier (see Box 1). Mutations found in FHL3 patients, leading to defective Munc13-4 protein, cause impaired degranulation and cytolytic activity in CTL and NK cells (Feldmann et al., 2003; Wood et al., 2009) (see Table 1). The conjugates of T cells from FHL3 patients show normal MTOC polarization and docking (i.e. close association of lytic granules with the plasma membrane). However, fusion of lytic granules with the plasma membrane and release of granule content into the synaptic cleft were not observed in these conjugates, suggesting a role of Munc13-4 in some of the terminal steps of exocytosis, such as tethering, priming and membrane fusion (Feldmann et al., 2003). The essential role of the Rab27a–Munc13-4 interaction in lytic granule exocytosis was revealed in CTLs obtained from FHL3 patients. There, wild-type Munc13-4, but not Munc13-4 comprising point mutations in the RBD or Munc13-4 lacking an RBD, was able to rescue degranulation (Elstak et al., 2011). Like cytotoxic lymphocytes, mast cells also release biologically active molecules through exocytosis of lysosomal organelles; therefore, Elstak and co-authors also assessed whether interaction between Rab27a and Munc13-4 is required for granule exocytosis in mast cells. Complementation assays performed after Munc13-4 knockdown in the rat mast cell line RBL-2H3 produced results similar to those

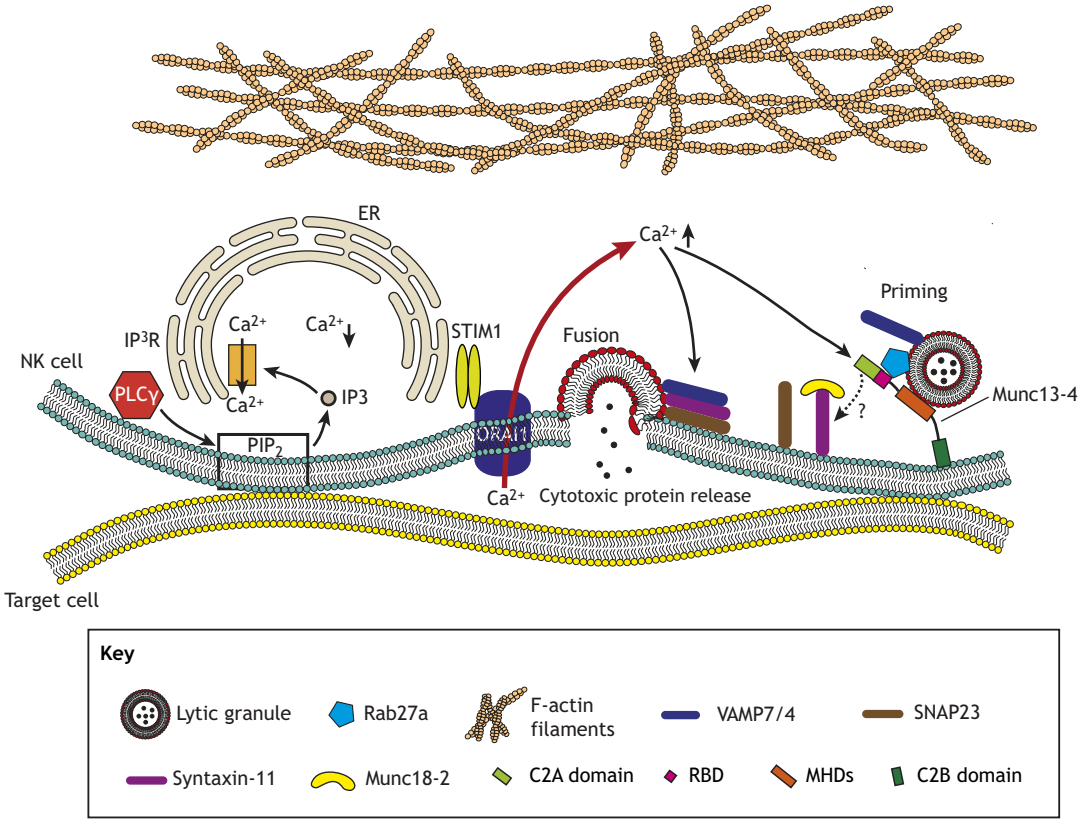

Fig. 3. Ca^{2+} mobilization, priming and fusion of lytic granules with the plasma membrane. PLCγ hydrolyses plasma membranous PIP_2 to diacylglycerol and IP_3, and binding of IP_3 to its receptor, a Ca^{2+} channel on the ER triggers the release of Ca^{2+} from the ER into the cytosol. This Ca^{2+} depletion activates STIM1 in the ER membrane, which opens the Ca^{2+} channel ORAI1, enabling entry of extracellular Ca^{2+} into the cell in a process called store-operated Ca^{2+} entry (SOCE). The increase in intracellular Ca^{2+} concentration is sensed by the C2 domains (C2A and C2B) of Munc13-4. The C2A domain is known to interact with different syntaxins in immune and non-immune cells in a Ca^{2+}-dependent manner to prime vesicle fusion with the plasma membrane. Munc13-4 also binds STX11; however, the role of this interaction in the priming process has not yet been investigated in NK cells (indicated by question mark). Priming is a Ca^{2+}-dependent process that involves assembly of trans-protein complexes comprising SNARE proteins located on vesicles (v-SNAREs) and those on the plasma membrane (t-SNAREs). Priming of lytic granules through trans-protein complex assembly between t-SNAREs, STX11 and SNAP23, and v-SNAREs, VAMP4 and/or VAMP7, mediated by STXBP2, leads to the fusion of lytic granules with plasma membrane and release of their lytic content into the synaptic cleft.

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Box 1. Munc13-4 in lytic granule maturation

In non-activated human CTLs and NK cells, Rab27a and Munc13-4 are located on disparate vesicles distinct from perforin-containing lytic granules (Menager et al., 2007; Wood et al., 2009): Rab27a colocalizes with Rab7 on late endosomes, whereas Munc13-4 colocalizes with Rab11 on recycling endosomes. In primary human CTLs, Munc13-4 mediates assembly of a late endosome and a recycling endosome into a common endosomal vesicle that is independent of Munc13-4 binding to Rab27a and the activation status of the cell (Menager et al., 2007). Upon activation, these endosomal vesicles localize to the immune synapse in a way that is independent of lytic granules and only fuse with lytic granules near the plasma membrane (Menager et al., 2007). Similarly, in human NK cells, Rab27a and Munc13-4 colocalize with lytic granules upon activation; however, unlike in T cells, this recruitment does not happen near the synapse but at centrally located MTOCs within the cell (Wood et al., 2009). Recruitment is mutually dependent – Rab27a fails to localize to lytic granules in Munc13-4-deficient NK cells and vice versa (Wood et al., 2009). In human NK cells, recruitment of Rab27a and Munc13-4 to lytic granules is preferentially regulated by activation of different receptors. CD16, whose activation can induce degranulation in resting NK cells, recruits Munc13-4 to lytic granules. LFA-1, NKG2D and 2B4, however, whose individual activation fails to induce degranulation, recruit Rab27a to lytic granules. Tandem activation of NKG2D and 2B4 can stimulate degranulation in NK cells, and recruits both Rab27a and Munc13-4 to lytic granules (Wood et al., 2009). The preferential recruitment of Munc13-4 to lytic granules by CD16 points to the preeminent role and indispensability of Munc13-4 in exocytosis of lytic granules. This is further supported by total abrogation of degranulation observed in NK cells from FHL3 patients (Wood et al., 2009). Although Rab27a deficiency in Griscelli syndrome type 2 patients also led to diminished degranulation, some NK cells were still able to degranulate at low levels (Wood et al., 2009). The less-severe phenotype observed in Rab27a-deficient NK cells could be the result of Rab proteins being redundant as binding partners. Munc13-4 has been shown to bind to Rab11 (Johnson et al., 2016) and Rab15 (Zografou et al., 2012) in non-immune cells. Inactivation of Rab11-bearing recycling endosomes led to a slight but insignificant decrease in human NK cell degranulation (Reefman et al., 2010), indicating a marginal, if any, role of Rab11 in the exocytosis of lytic granules within NK cells.

observed in CTLs from FHL3 patients. These findings demonstrate a general role of Munc13-4 regarding granule exocytosis in immune cells (Elstak et al., 2012, 2011).

In non-activated RBL-2H3 cells, motile granules are found at the plasma membrane and their mobility decreased upon activation, suggesting increased entrapment and corralling of the granules at dthe membrane. However, no such motility decrease after activation was observed in cells expressing Munc13-4 with the aforementioned point mutations, suggesting a requirement of the interaction between Munc13-4 and Rab27a for entrapment and/or tethering of granules at the plasma membrane (Elstak et al., 2011). A similar role of Munc13-4 in limiting mobility of Rab27a-positive granules at the plasma membrane of activated neutrophils, which also release their granules through exocytosis, has also been observed (Johnson et al., 2011). As mentioned above, Rab27a enhances movement of lytic granules near the plasma membrane in resting NK cells (Liu et al., 2010) and, given the findings in mast cells and neutrophils, interaction between Munc13-4 and Rab27a seems likely to arrest their mobility upon activation (Fig. 2). However, the role of Munc13-4 in tethering lytic granules to immune synapses has been shown to be redundant in mouse CTLs: there, Munc13-4 and Munc13-1 are expressed, and the defective tethering phenotype of Munc13-4-defective CTLs could be rescued by Munc13-1 (Dudenhöffer-Pfeifer et al., 2013). In fact, knockdown of Munc13-1 further reduced already diminished degranulation in Munc13-4-deficient mouse CTLs, thus, suggesting a physiological role of Munc13-1 in mouse CTL degranulation (Dudenhöffer-Pfeifer et al., 2013). Significantly diminished degranulation in CTLs (Feldmann et al., 2003) and total abrogation of degranulation in NK cells from FHL3 patients (Wood et al., 2009) rule out such a redundancy in human lymphocytes, particularly NK cells, and also underscore the difference between mouse and human lymphocytes. In summary, Rab27a anchors lytic granules to the plasma membrane through its effectors, mainly Munc13-4, during docking and tethering steps of the lymphocyte cytotoxicity.

Ca^{2+} mobilization during degranulation

Like neural and endocrine cell exocytosis, degranulation in NK cells is also Ca^{2+} dependent (Galandrini et al., 2013; Tuosto et al., 2015). NK cell activation leads to an increase in intracellular Ca^{2+}; this is initially triggered by release of Ca^{2+} from intracellular stores, followed by influx of extracellular Ca^{2+} across the plasma membrane (Leibson et al., 1990). Phospholipase Cγ (PLCγ) is required for this Ca^{2+} mobilization in activated NK cells and their cytolytic function (Billadeau et al., 2003; Caraux et al., 2006; Tassi and Colonna, 2005; Tassi et al., 2005). Of the two PLCγ isoforms PLCγ1 and PLCγ2, the latter seems to play a non-redundant role as the former only partially rescues cytotoxicity of PLCγ2-deficient mouse NK cells (Regunathan et al., 2006). Furthermore, PLCγ2 is exclusively required downstream of NKG2D receptor signaling in NK cells (Upshaw et al., 2005). PLCγ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane into diacylglycerol and inositol 1,4,5-trisphophate (IP_3), the latter binding to the IP_3 receptor, a Ca^{2+} channel on the endoplasmic reticulum (ER), triggers release of Ca^{2+} from the ER into the cytosol (Gumbleton and Kerr, 2013). Depletion of Ca^{2+} from the ER activates stromal interaction molecule 1 (STIM1), a protein located in the ER membrane, which opens the Ca^{2+} release-activated Ca^{2+} channel ORAI1 to enable entry of extracellular Ca^{2+} intro the cell in a process called store-operated Ca^{2+} entry (SOCE) (Galandrini et al., 2013; Gumbleton and Kerr, 2013) (Fig. 3). The dependence of NK cell cytotoxicity on SOCE was confirmed by the impaired cytolytic function observed in NK cells from patients with mutations in either STIM1 or ORAI1 and in SOCE inhibitor-treated NK cells from healthy individuals (Maul-Pavicic et al., 2011). Also, normal granule polarization to the synapse but impaired degranulation in SOCE-blocked NK cells required Ca^{2+} for the terminal steps of lytic granules exocytosis (Maul-Pavicic et al., 2011). This is in contrast to mouse NK cells in STIM1 and STIM2 double knockout mice, which show normal degranulation and cytotoxicity (Freund-Brown et al., 2017).

PIP_2 in lytic granule exocytosis

PIP_2, the source of IP_3, is generated from phosphatidylinositol 4-phosphate (PI(4)P) by phosphorylating the D5 position of its inositol ring, which is catalyzed by phosphatidylinositol 4-phosphate-5-kinase (PIP5K) (Tuosto et al., 2015). Visualization of PIP_2 – using a GFP-tagged pleckstrin homology (PH) domain of PLCγ1 (GFP-PH) – showed progressive reduction of its levels at the area of immune synapse but not in the membrane away from the synapse, thereby, revealing its utilization at the NK-target contact site (Micucci et al., 2008). GFP-PH can also act as an inhibitor of PIP_2-dependent processes, and GFP-PH-expressing cells showed significantly reduced cytolytic activity and degranulation in response to activation of different activating receptors,

demonstrating requirement of PIP_2 for the exocytosis of lytic granules (Micucci et al., 2008).

Of the three isoforms of PIP5K, PIP5Kα and PIP5Kγ are expressed in human NK cells, whereas PIP5Kβ is almost undetectable (Micucci et al., 2008). In human NK cells, activation of the FCGR3A (hereafter referred to as CD16) receptor recruits PIP5Kα to the plasma membrane in an Arf6 (a small G-protein of the Ras superfamily)-dependent way to generate PIP_2 (Galandrini et al., 2005). Individual silencing of PIP5Kα and PIP5Kγ in NK cells results in reduced PIP_2 levels, and impaired degranulation and cytotoxicity without affecting conjugate formation and granule polarization (Micucci et al., 2008). PIP5K silencing reduced IP_3 generation, without having any effect on the phosphorylation levels of PLCγ, an indicator of its activation status. This suggests that decreased PLCγ activity is not due to impaired activation receptor signaling but probably a result of reduced PIP_2 substrate availability (Micucci et al., 2008). These findings indicate that, in activated NK cells, PIP_2 generated by PIP5K is hydrolyzed by PLCγ to generate IP_3, which, through SOCE, increases intracellular Ca^{2+} levels to drive lytic granule fusion (Fig. 3).

In neurons, synaptotagmin I (Syt1) acts as Ca^{2+} sensor to regulate neurotransmitter release (Fernández-Chacón et al., 2001) and binds Ca^{2+} through its two C2 domains, C2A and C2B (Shin et al., 2009). When expressed in mouse CTLs and localizing to lytic granules, the synaptotagmin family member synaptotagmin VII (Syt4) might serve a similar role in cytotoxic lymphocytes (Fowler et al., 2007). Despite normal conjugate formation and granule polarization to the synapse, CTLs from Syt4-deficient mice show impaired cytolytic activity, thereby suggesting that Syt4 regulates the terminal steps of lytic granule exocytosis (Fowler et al., 2007). Another candidate that might act as a Ca^{2+} sensor is Munc13-4. Like Syt1 and Syt4, it contains Ca^{2+}-binding domains, and Munc13-4 constructs that either contain a C2A or C2B domain, or both domains, are unable to rescue defective degranulation and cytotoxicity in NK cells (Bin et al., 2018). Specifically, the C2 domains of Munc13-4 influence both the efficacy and Ca^{2+} sensitivity of degranulation in NK cells (Bin et al., 2018). In summary, degranulation in NK cells requires an increase in intracellular Ca^{2+} mediated through SOCE, which is triggered by IP_3 generated through PLCγ-mediated hydrolysis of PIP_2. It remains to be demonstrated, but Munc13-4 is likely to be the Ca^{2+} sensor in NK cells.

Priming and fusion of lytic granules with the plasma membrane

In regulated vesicle exocytosis, the priming process renders vesicles fusion competent (Klenchin and Martin, 2000). Priming involves assembly of a large trans-protein complex that comprises soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins located on vesicles (v-SNAREs) and the plasma membrane (t-SNAREs) (Tang, 2015). Munc13 family members facilitate the priming of synaptic vesicle in pre-synaptic neurons (Augustin et al., 1999a,b), whereas its fellow family member Munc13-4 is likely to serve a similar function in NK cells. Studies performed with v-SNARE and t-SNARE liposomes demonstrated the ability of Munc13-4 to promote trans-SNARE complex assembly and liposome fusion in a Ca^{2+}-dependent manner (Boswell et al., 2012). The C2A domain of Munc13-4 is required for its Ca^{2+}-dependent interaction with t-SNAREs, whereas the C2B domain mediates the Ca^{2+}-dependent interaction with phospholipids (Boswell et al., 2012). Munc13-4 has been shown to facilitate Ca^{2+}-regulated exocytosis in rat neuroendocrine pheochromocytoma (PC12) cells, platelets and RBL-2H3 mast cells and bind to their respective t-SNAREs, syntaxin-1, -2 and -4 (Boswell et al., 2012). It also binds to syntaxin-11 (STX11), which plays a crucial role in the exocytosis of lytic granules in cytotoxic lymphocytes (Boswell et al., 2012). Mutations in the *STX11* gene cause familial hemophagocytic lymphohistiocytosis, referred to as FHL4 (see Table 1). NK cells from FHL4 patients and STX11-silenced NK cells from healthy donors display impaired degranulation and cytotoxicity (Arneson et al., 2007; Bryceson et al., 2007), without affecting granule polarization (Bryceson et al., 2007). A similar impaired phenotype was also observed in NK cells from STX11-deficient mice (D'Orlando et al., 2013). In resting human NK cells, STX11 colocalizes with cation-dependent mannose-6-phosphate receptor – a marker of late endosomes and the trans-Golgi network (Lin et al., 2004) – that is distinct from Rab27a vesicles and lytic granules. However, STX11 partially colocalizes with both of them at the immune synapse in activated NK cells, suggesting its involvement in the terminal steps of exocytosis (Dabrazhynetskaya et al., 2012). Distinct endosomal location of STX11, away from lytic granules, was also observed in resting human CTLs and as observed in NK cells, STX11 colocalized with lytic granules only at the immune synapse. However, in contrast to resting NK cells, in resting CTLs it colocalizes with Rab11a, a marker of recycling endosomes (Halimani et al., 2014). In activated CTLs, recycling endosomes that carry STX11 reach and fuse with the immune synapse before lytic granule arrival, a process that is dependent upon the v-SNARE protein vesicle-associated membrane protein 8 (VAMP8) (Halimani et al., 2014; Marshall et al., 2015). It is currently unknown whether Munc13-4 – which colocalizes with Rab11 in recycling endosomes, and helps to mediate fusion between recycling endosomes and Rab27a-containing late endosomes in CTLs (Ménager et al., 2007) – has any role in the transport of STX11 to and its fusion of endosomes with the plasma membrane. However, given that Munc13-4 binds to STX11 (Boswell et al., 2012) this is worth investigating. Transport to and fusion of STX11-bearing vesicles with the immune synapse prior to the arrival of lytic granules, as well as significantly reduced lytic granule dwelling time and fusion with the immune synapse in STX11-silenced CTLs, provide strong evidence for its role as a t-SNARE in cytotoxic lymphocytes (Halimani et al., 2014).

Role of interaction between STX11 and STXBP2 in cytotoxicity of NK cells

In human NK cells, STX11 expression is differentially regulated by different protein degradation pathways. It is decreased by proteasome inhibition, increased by pan-caspase inhibition, whereas inhibition of lysosomal or tripeptidyl peptidase II pathway neither affects the steady-state levels nor the decrease induced by proteasome inhibition (Dabrazhynetskaya et al., 2012). Restoration of STX11 expression by inhibition of pan-caspase in proteasome inhibitor-treated NK cells suggests the existence of a STX11-interacting protein that can serve as a substrate for proteasomal degradation but might target STX11 to caspase-mediated degradation (Dabrazhynetskaya et al., 2012). This regulation is unique to NK cells, as proteasome inhibition barely affects STX11 expression in CTLs (Dabrazhynetskaya et al., 2012). Syntaxin protein expression is also stabilized by interaction of NK cells with Munc18 proteins, which act as their chaperones (Deshpande and Rodal, 2016). In NK cells, STX11 interacts with syntaxin-binding protein 2 (STXBP2; also known as Munc18-2) and mutations in STXBP2, leading to low or no expression in familial hemophagocytic lymphohistiocytosis (FHL) variant 5 (FHL5; see

Table 1) patients result in identical changes in the expression of its binding partner STX11 (Côte et al., 2009; zur Stadt et al., 2009). Similarly, mutations in the N-terminal region of STX11 that disable its binding to STXBP2 and are found in FHL4 patients, diminish STX11 expression (Müller et al., 2014). NK cells from FHL5 patients phenocopy those obtained from FHL4 patients, showing defective degranulation and cytotoxicity without impairment of granule polarization, thereby underlining the functional requirement of interaction between STX11 and STXBP2 for the terminal step of degranulation (Côte et al., 2009; zur Stadt et al., 2009). STXBP2 not only stabilizes the expression of STX11, but is also required for STX11 localization to the plasma membrane in human CTLs (Dieckmann et al., 2015). Also, in FHL5 patients, mutations that do not affect binding of STXBP2 to STX11 and, therefore, expression of STXBP2, nevertheless impair degranulation and cytotoxicity of T and NK cells (Spessott et al., 2015). STXBP2 promotes assembly of the trans-SNARE complex between STX11 and other SNAREs, as well as subsequent membrane fusion in human CTLs (Spessott et al., 2017, 2015). Impaired degranulation and cytotoxicity of NK cells from FHL4 and FHL5 patients can be partially restored by treatment with interleukin 2 (IL2) (Bryceson et al., 2007; Côte et al., 2009; zur Stadt et al., 2009). IL2, an NK cell cytotoxicity-enhancing cytokine (Becknell and Caligiuri, 2005), activates expression of surrogate proteins syntaxin-3 (STX3) and STXBP1 in cytotoxic lymphocytes (Hackmann et al., 2013). STXBP2 binds to STX3 and, in the absence of STX11, delivers it to plasma membrane of human CTLs, and STXBP1 binds both STX11 and STX3, thereby suggesting the presence of alternative syntaxin-STXBP pairings that may be employed in IL2-activated cytotoxic lymphocytes from FHL4 and FHL5 patients (Hackmann et al., 2013). The presence of alternative pairings between syntaxin and STXBP has also been supported by a recent study, investigating T and NK cells from FLH5 patients that carry STXBP2 mutations (Lopez et al., 2018). Surprisingly, reduced expression of STXBP1 and its cognate t-SNARE syntaxin-1 were found, in addition to the expected diminished expression of STXBP2 and STX11. Further inquiry into the role of STXBP1 showed significantly reduced cytotoxicity in a STXBP1-silenced NK cell line (Lopez et al., 2018). It is not known whether syntaxin-1 also plays any role in the degranulation of NK cells, as STXBP1 also binds STX11 in cytolytic lymphocytes (Hackmann et al., 2013). Syntaxin-7 has been shown to be required for degranulation in human CTLs (Pattu et al., 2011); however, its role in NK cells has not been studied.

In summary, impaired degranulation and cytotoxicity in FHL4 and FHL5 patients clearly establish the requirement of the interaction between STX11 and STXBP2 in cytotoxic lymphocytes, wherein STXBP2 stabilizes STX11 expression and facilitates trans-SNARE complex assembly between STX11 and other SNAREs. Expression of surrogate syntaxins and STXBP, and their interactions indicate the existence of alternative pairings between syntaxin and STXBP that may mediate the function of STX11 bound to STXBP2 in their absence; however, more investigations are necessary to establish such a role unambiguously.

Assembly of the trans-SNARE complex during degranulation

Syntaxins are not the only t-SNAREs that are involved in assembly of the trans-SNARE complex in T and NK cells. STX11 has been shown to interact with synaptosomal-associated protein 23 (SNAP23) in both human NK and CTLs (Hellewell et al., 2014; Spessott et al., 2017). The v-SNAREs implicated in degranulation within cytotoxic lymphocytes include VAMP2, VAMP4, VAMP7 and VAMP8, and the protein vesicle transport through interaction with t-SNAREs 1B (VTI1B) (Chitirala et al., 2019; Dressel et al., 2010; Krzewski et al., 2011; Loo et al., 2009; Marcet-Palacios et al., 2008; Marshall et al., 2015; Matti et al., 2013; Qu et al., 2011). In mouse CTLs, VAMP2 and VAMP8 colocalize with lytic granules, whereas CTLs from VAMP2- and VAMP8-deficient mice display impaired degranulation and cytotoxicity (Dressel et al., 2010; Loo et al., 2009; Matti et al., 2013). Impaired degranulation has also been observed in VTI1B-deficient mouse and human CTLs (Dressel et al., 2010; Qu et al., 2011). In contrast to impaired degranulation of mouse CTLs in response to tetanus toxin (Matti et al., 2013), which selectively cleaves VAMP1, VAMP2 and VAMP3 but not VAMP4, VAMP7 and VAMP8, human CTLs are insensitive to tetanus toxin effect (Chitirala et al., 2019), highlighting the inter-species difference in mechanisms involved in degranulation. In human CTLs, STXBP2 facilitates trans-SNARE complexes between STX11, SNAP23 and VAMP8 to promote membrane fusion (Spessott et al., 2017). A recent study also demonstrated the requirement of VAMP7 for the fusion of lytic granules with the plasma membrane and the formation of the trans-SNARE complex comprising VAMP7, SNAP23 and STX11 in human CTLs (Chitirala et al., 2019) (Fig. 3). The v-SNAREs that are known to be required for NK cell degranulation are VAMP4 and VAMP7 (Krzewski et al., 2011; Marcet-Palacios et al., 2008). Both proteins colocalize with lytic granules at the immune synapse, and individual knockdown of these proteins inhibits degranulation and cytolytic activity of human NK cells. Compared with VAMP4 and VAMP7, VAMP1, VAMP3 and VAMP8 show minimal colocalization with lytic granules in activated NK cells, suggesting a lack of requirement in NK cell degranulation (Krzewski et al., 2011). In summary, lytic granules docked and tethered to the immune synapse are primed – most probably through the interaction between Munc13-4 and STX11 – to form the trans-SNARE complex comprising t- and v-SNAREs. On the basis of the available evidence, t-SNAREs, STX11 and SNAP23 together with v-SNAREs, VAMP7 and VAMP8 in CTLs, and with VAMP4 and VAMP7 in NK cells, are likely to be the components of the trans-SNARE complex in cytotoxic lymphocytes, assembly of which is probably mediated by STXBP2 (Fig. 3).

Endocytosis of lytic granules for serial killing

After the fusion of lytic granules at the synaptic cleft, the lytic granule membrane proteins are endocytosed in order to replenish lytic granules for subsequent rounds of killing. This enables cytotoxic lymphocytes to mediate the elimination of multiple targets through successive contact and degranulation within a short period of time, a phenomenon termed serial killing (Bhat and Watzl, 2007; Chang et al., 2016; Martz, 1976). Endocytosis of the lytic granule v-SNARE

Table 2. Difference between CTLs and NK cells

Cellular process	CTL	NK cell
Colocalization of Rab27a and Munc13-4 with lytic granules	At the immune synapse	Away from the synapse at centrally located MTOC
Endosomal location of STX11 in inactivated cells	Recycling endosome	Late endosome
Expression of STX11	Not affected by proteasome inhibition	Decreased by proteasome inhibition
Vesicle SNAREs involved in trans-SNARE complex formation	VAMP7 and VAMP8	VAMP4 and VAMP7

VAMP2 has been demonstrated in mouse CTLs following its contact with the target cell. This was achieved employing live cell time-lapse confocal imaging following the addition of a fluorochrome-conjugated antibody to the extracellular medium, which binds VAMP2 only upon fusion of lytic granules with the plasma membrane and the subsequent externalization of VAMP2 on the plasma membrane surface (Chang et al., 2016). Pharmacological and genetic studies have shown that endocytosis occurs through clathrin-coated pits, whose scission from the plasma membrane is dependent on dynamin, a GTPase involved in the generation of nascent endocytic vesicles (Chang et al., 2016). Significantly, dynamin-2 was found to localize with lytic granules to the cytotoxic synapse formed between human NK cells and the EBV-transformed human B cell line 721.221, and knockdown of dynamin-2 or pharmacologic inhibition of dynamin was shown to impair release of lytic granules and cytotoxicity of NK cells (Arneson et al., 2008). The endocytic vesicle is recycled through early and late endosomes but not recycling endosomes and, at late-endosome stage, granzyme B is loaded to regenerate mature lytic granules. When endocytosis of lytic granule proteins is blocked through inhibition of dynamin, the serial killing by mouse CTLs is severely impaired (Chang et al., 2016). The vesicle-associated protein Ca^{2+} channel flower homolog (CACFD1) mediates endocytosis of lytic granule membrane proteins in a Ca^{2+}-dependent manner (Chang et al., 2018). Dynamin- and clathrin-dependent endocytosis of lytic granule-associated Munc13-4 has also been reported in primary human NK cells, and also requires PIP_2 generated by PIP5Kγ (Capuano et al., 2012). Blocking endocytosis through PIP5Kγ silencing reduces the killing frequency of human NK cells, indicative of their impaired serial killing ability (Capuano et al., 2012). Thus, dynamin-dependent endocytosis in clathrin-coated vesicles recycles lytic granule proteins to regenerate mature lytic granules that enable cytotoxic lymphocytes to kill multiple target cells in a short period of time.

Conclusions and perspectives

Investigations into the genetic determinants and functional impairment of cytotoxic lymphocytes in different immune disorders, particularly FHL (see Table 1), have elucidated the molecular regulation of plasma membrane-proximal cellular steps involved in the cytolytic function of NK cells. Roles of molecular players, such as myosin IIa, Rab27a, Munc13-4, STX11 and STXBP2 are well-established during the terminal steps of NK cell degranulation (Figs 1–3). However, there are many gaps in our understanding. For example, regulators of Rab27a activation and deactivation during NK cell killing, i.e. Rab27a guanine nucleotide exchange factors (GEFs) and Rab27 GTPase activating proteins (GAPs), respectively, are still unknown. The components of the signaling pathways that mediate activation of Rab27a have also not yet been identified. Moreover, it has not been determined whether Munc13-4 tethers lytic granules to the plasma membrane only through its C2 domains or are whether there are other proteins it interacts with in order to perform this function. As stated above, T and NK cells share the mechanism and molecular machinery of degranulation. However, not all molecular requirements are identical between the two cell types (Table 2) and the discoveries in T cells need to be validated in NK cells. Also, differences exist between mouse and human NK cells; this underscores the risk in extrapolating findings in mouse NK cells to human NK cells. With the increasing interest in NK cells as a treatment option against cancer, a detailed molecular understanding of the mechanisms underlying the cellular processes involved in NK cytolytic function may help improve the effectiveness of NK cell immunotherapy.

Competing interests

The authors declare no competing or financial interests.

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