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Navigating Barriers: The Challenge of Directed Secretion at the Natural Killer Cell Lytic Immunological Synapse

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Abstract

Introduction—Natural killer (NK) cells have an inherent ability to recognize and destroy a wide array of cells rendered abnormal by stress or disease. NK cells can kill a targeted cell by forming a tight interface—the lytic immunological synapse. This represents a dynamic molecular arrangement that over time progresses through a series of steps to ultimately deliver the contents of specialized organelles known as lytic granules.

Discussion—In order to mediate cytotoxicity, the NK cell faces the challenge of mobilizing the lytic granules, polarizing them to the targeted cell, facilitating their approximation to the NK cell membrane, and releasing their contents.

Conclusion—This review is focused upon the final steps in accessing function through the lytic immunological synapse.

Keywords

NK cell; cytotoxicity; myosin IIA; immune synapse

Introduction

Natural killer (NK) cells are the major lymphocytes of the innate immune system that provide critical defense against cancerous and virally infected cells. NK cells are activated by the ligation of germline-encoded receptors, which do not require genetic recombination events to attain specificity. Upon recognizing target cells using these receptors, NK cells promote host defense by lysing the target cells via the directed secretion of lytic granules. Lytic granules are specialized organelles, a form of secretory lysosomes, containing the pore-forming molecule perforin and cell death-promoting molecules such as granzymes. After activation, NK cells also secrete cytokines and provide costimulation to other immune cells to promote an effective immune response (reviewed in [1]). This review will focus on NK cell lytic granule-mediated cytotoxicity, with a special focus on the final steps of secretion.

NK cell activation takes place in a tightly regulated series of stages. When an NK cell encounters a transformed or infected target cell, receptors strengthening the interaction between the effector and target cells are ligated. Selectins and integrins, such as L-selectin, LFA-1, and MAC-1, have been implicated in the initial adhesion step and are required for effective conjugation between NK cells and their targets [2–5]. This adhesion allows for activation receptors on the NK cell, such as natural cytotoxicity receptors and activating killer cell immunoglobulin-like receptors (KIRs), to focus in the direction of the target cell. In NK cells, activation signaling can be balanced by inhibitory signaling, often from inhibitory KIRs recognizing self major histocompatibility molecules [6]. If the balance of activation and inhibition leans towards activation, the NK cell can progress through further steps in maturing a lytic interface with its target and thus initiate steps to kill the dangerous cell.

Following adhesion and activation signaling, the NK cell forms a tight interface with the target cell, known as the immunological synapse (IS). The IS is a site of actin polymerization and reorganization [7], which require Vav1 GEF activity, actin nucleation by Arp2/3 and WASp, and stabilization of nascent filaments by HS1 [8–11]. Without the ability to reorganize F-actin, NK cells are not able to focus receptors to the interface and polarize lytic granules toward the IS for secretion [3,9,12,13]. This suggests that F-actin polymerization at the IS represents an early step in the process of cytotoxicity. Once F-actin polymerization at the IS begins, the contact site organizes into several discrete zones, including a ring of F-actin and integrins at the periphery, known as the peripheral supramolecular activation cluster (pSMAC), and a cluster of critical signaling molecules at the central region of the interface, known as the central supramolecular activation cluster (cSMAC). Although the purpose of organization into the pSMAC and cSMAC is debated and likely differs between cell types, proper organization of the synapse is thought to be required for directed secretion of lytic granules in NK cells [14, 15].

Once an organized IS is formed, adhesion and activation signaling are further amplified, finally reaching an activation threshold which directs the NK cell to commit to secreting lytic granule contents. In order for this form of directed secretion to occur, the microtubule organizing center (MTOC) and lytic granules must polarize to the IS. Movement of the MTOC to the IS is known to depend upon ERK, Vav1, Pyk2, and hDial1 [9,13,16,17], and stabilization of the MTOC and at the IS depends upon CIP4 [18] but details of this movement are still unclear. One important signal for granule polarization to the IS in NK cells appears to be that from integrins [15], although in T cells, this may not be necessary [19]. In T cells, the movement of lytic granules and the MTOC to the IS is thought to occur in two possible ways, which are dependent upon strength of signal [19,20]. When a strong signal is received, lytic granules are delivered quickly to the IS in a focused manner, but when altered peptide ligands for the T cell receptor are used experimentally, polarization is less focused. Whether NK cells use these methods of granule delivery to the IS remains unknown; however, recent evidence from our laboratory defines a rapid approximation of lytic granules to the MTOC prior to their delivery to the IS (Mentlik AN, Sanborn KB, Holzbaaur EL, and Orange JS, submitted). In this work, the movement of lytic granules along microtubules was shown to be dynein-dependent, thus demonstrating dynein as a minus-ended microtubule motor for lytic granules, suggesting that dynein directly moves the granules along microtubules toward the MTOC. The lysosomal sorting protein AP3 is also required for granule movement to the IS [21], likely due to a role in sorting a protein required for movement rather than a direct motor function.

Once the lytic granules reach the IS, their contents are exocytosed through a process of vesicle priming, docking, and membrane fusion (discussed below). The directed secretion of granule contents, including the pore-forming molecule perforin and cell-death promoting molecules such as granzymes, directs the organized death of the infected or transformed target cell. There may, however, be additional obstacles for polarized granules to overcome prior to priming,

docking, and fusion with the plasma membrane. In activated NK cells, the F-actin cortex at the IS is dense and may present a barrier to secretion. Several questions remain regarding this step of directed secretion: How do NK cell lytic granules reach the plasma membrane upon arriving at the IS? How is F-actin cleared away for secretion, and is this a necessary component for secretion? If so, are the clearances sufficient for granule fusion with the membrane, or are additional motor molecules required? Several recent findings have begun to mold our understanding of this part of the process and provide pieces to the complex puzzle of lytic granule exocytosis.

What Happens after a Lytic Granule is Polarized Towards the Immunological Synapse?

Once lytic granules are delivered to the IS by the microtubule network, they encounter a dense F-actin cortex. An effective activating IS in NK cells requires extensive actin polymerization, and without this polymerization, the MTOC and lytic granules do not polarize in the direction of the target cell [3,9,12]. Although precise measurements of the actin gathered at the IS have not been performed, fluorescent images indicate that this layer of actin is dense [3,22,23]. This amount of actin could act as a substantial physical barrier to the secretion of lytic granule contents. Granules may, therefore, require a combination of actin clearance mechanisms and additional, F-actin-based, motor molecules to reach the plasma membrane.

One potential mechanism for allowing granules access to the membrane is the creation of large clearances in F-actin. Similar to T cells, F-actin clearances in the cSMAC region of the IS have been reported in NK cells [3,5,24,25]. While the formation of the actin clearance is known to be independent of microtubule integrity and MTOC polarization [3], it has not yet been shown whether actin hypodensity in the cSMAC occurs as a result of symmetrical actin reorganization in the pSMAC region or if the clearances are actively created by bundling or depolymerizing actin filaments in the cSMAC. While several proteins, including the actin-associating protein HS1 and the activation receptor NKG2D, have been implicated in IS symmetry during cell spreading [11,26], in cells with perturbed actin dynamics, a central clearance is typically still evident. Additionally, it remains unknown whether any clearance in F-actin is long-lasting, or simply a transient effect during initial NK cell/target cell conjugation.

Recent data demonstrate that lytic granules reach the IS through travel with the MTOC [27 and Mentlik AN, Sanborn KB, Holzbaur EL, and Orange JS, submitted]. While there is evidence that granules reach the plasma membrane in T cells via their association with the MTOC and do not require any additional form of microtubule- or actin-based transport [27], it remains unclear whether all lytic granule transport to the IS from the MTOC is a feature of intimate MTOC approximation to the IS and independent of non-MTOC-associated motor molecules. NK cell lytic granule transport has been shown to be capable of occurring in the direction of microtubule plus-ends using the microtubule motor kinesin [28] and has been shown to require the actin motor myosin IIA [24,29]. This suggests that NK cell degranulation may require motor molecules following polarization of granules to the IS.

If large clearances in actin are, in fact, the location of secretion for lytic granule contents, it remains unclear whether lytic granules are delivered directly to these clearances, or whether they must travel along actin filaments at the IS to reach an actin-sparse region in which they might reach the plasma membrane. A recent study has shown that in CD4⁺ CTLs, lytic granules are initially delivered to the pSMAC region, while secretion of their contents occurs in the cSMAC [19]. The study did not elucidate the mechanism of transporting granules from the pSMAC to the cSMAC. Therefore, it is unclear whether the lytic granule movement requires travel along actin filaments or whether it is entirely microtubule-based. While delivery of granules to the pSMAC has not been demonstrated in NK cells, in T cells, signal strength has been shown to play a role in which method of transport lytic granules use to reach the IS [19, 20]. The strength of activation signaling vs. inhibitory signaling could, therefore, determine

whether NK cell lytic granules are secreted in a region that is replete or relatively devoid of F-actin. Irrespective, the physical challenge of the transit of a lytic granule of approximately 500 nm in size through the cell cortex is one which must, by necessity, require a solution. The process may utilize different mechanisms at different points during IS formation, at least some of which are likely to involve significant interaction with actin filaments.

Recently, a role for the actin motor protein myosin IIA has been shown in NK cell cytotoxicity [24,29]. In primary human NK cells and human NK cell lines treated with the myosin II inhibitor blebbistatin or siRNA against myosin IIA (but not myosin IIB), cytotoxicity was significantly decreased. Although NK cells without myosin IIA function were capable of conjugating with target cells, polymerizing actin at the IS, and polarizing activation receptors, the MTOC, and lytic granules to the IS, they were unable to degranulate [24]. Additionally, IFN- γ secretion was normal in NK cells following myosin IIA inhibition, suggesting a specific role for myosin IIA in degranulation [29]. Examination of NK cells from patients with a truncation mutation in myosin IIA (1933x) revealed a similar specific decrease in cytotoxicity, while conjugation and IS formation occurred normally [29]. Myosin IIA was found in preparations of enriched lytic granules from NK cell lines as well as primary NK cells, including those from patients with the 1933x myosin mutation. Furthermore, myosin IIA was directly noted at the surface of lytic granules by high-resolution ultrastructural studies and was confirmed biochemically through the surface biotinylation of lytic granules. The association between myosin IIA and lytic granules was functionally relevant, as lytic granules isolated from human cell lines and control donor primary NK cells were capable of binding to F-actin in vitro. Granules from patients with the 1933x mutation, as well as control granules treated with blebbistatin, were incapable of adhering to F-actin, suggesting roles for the actin motor function and C terminus of myosin IIA. There is a likely role for myosin IIA in directing granule transport, as decreased granule introduction into and motility in the cell cortex was observed by total internal reflection fluorescence microscopy following inhibition of the myosin light chain kinase [29]. As a result, the data suggest that myosin II motor function physically guides the motion of granules in relation to the actin network at the IS.

This role for myosin IIA in NK cell degranulation suggests that NK cells require actin-based motor function for the transport of lytic granules across F-actin. The nature of this requirement, however, is unclear and depends largely upon whether NK cells secrete lytic granule contents through large actin clearances or through smaller conduits in the actin network. Myosin IIA may have a role in the movement of lytic granules from the actin-dense pSMAC to large clearances at the cSMAC, allowing for the lytic granules to reach the plasma membrane upon reaching an actin clearance. Alternatively, if lytic granules use smaller actin conduits rather than the cSMAC for secretion, the requirement for myosin IIA may be in directing the granules through these conduits. Another possibility is that myosin IIA is directly involved in the creation of the actin conduits themselves. Large-scale remodeling of actin at the IS in NK cells treated with blebbistatin was normal [24]; however, if myosin IIA is required for the creation or enlargement of small actin conduits, this could represent a novel function for this actin-based motor. Defining this function will likely require advanced imaging techniques, given the small size of the structures in question. Further studies defining how lytic granules interact with F-actin will additionally clarify the role of actin-interacting and actin motor proteins in NK cell granule localization and degranulation.

Other questions remain about the precise mechanism by which myosin IIA acts in regulating degranulation in NK cells. Although the protein has been shown to localize to the outer membrane of lytic granules [29], it is not known whether this association with the membrane is direct or whether myosin IIA interacts with other proteins on the granule membrane. Additionally, the stoichiometry of the myosin IIA association with lytic granules remains unclear. Although myosin IIA could interact with lytic granules as an individual dimer using

its cargo domain (Fig. 1a), the structure of myosin IIA makes it unlikely to be able to facilitate organelle walking similar to myosin V [30,31]. Furthermore, an association between myosin IIA and lytic granules was still observed in patients harboring a 1933x truncation, suggesting an alternative model, specifically one in which myosin IIA forms filaments to wrap around NK cell lytic granules (Fig. 1b). This orientation could exploit the contractile nature of myosin filaments to guide lytic granule movement through the F-actin cortex at the IS. It is also unclear how myosin IIA function is specifically controlled at or confined to the IS, given that myosin IIA is constitutively associated with NK cell lytic granules [29]. Because myosin IIA is not recruited to granules once they arrive at the IS, its function in degranulation is likely to be regulated by proteins specifically localized there. Regulation of phosphorylation states is a probable mechanism, as myosin IIA filament stability and contractility are regulated by phosphorylation of its non-helical tailpiece [32]. While myosin IIA is involved in the final steps of granule approximation to the IS, there are clearly numerous unanswered questions that will define the precise role of this motor in directing cytotoxic function.

Accessing Cytotoxicity from the Synaptic Membrane-Approximated Lytic Granule

Once NK cell lytic granules gain access to the plasma membrane, they must fuse with the membrane to allow for the secretion of their contents. This appears to be a multistep process, involving tethering, vesicle priming, and membrane fusion. In CTLs, the small GTPase rab27a has been shown to link lytic granules as cargo to their target membranes [33]. This tethering step appears to be necessary for granule approximation to the plasma membrane. Once granules are tethered to the membrane, they must be prepared for fusion. Munc13-4, a binding partner of rab27a, has a critical role in vesicle priming for lytic granule exocytosis in CTLs and mast cells [34], and although levels of Munc13-4 are reduced in NK cells compared to T cells [34], the protein has been shown to interact with rab27a in NK cells as well [35]. Fusion of lytic granules with the membrane is regulated by soluble *N*-ethylmaleimide-sensitive protein attachment protein receptor (SNARE) proteins, which target granules to the correct portion of the membrane for fusion. SNARE-related proteins required for NK cell cytotoxicity include VAMP7, which associates with lytic granules [36], as well as syntaxin-11 and Munc18-2, which interact with each other to drive membrane fusion for lytic granule exocytosis in NK cells [37–39]. These proteins are defective in a series of human diseases resulting from impaired degranulation (reviewed in [40]). The proteins which mediate granule tethering, priming, and membrane fusion may be differentially recruited depending on strength or type of activation signal, serving as an additional layer of regulation for the process of exocytosis [41].

Once lytic granules fuse with the plasma membrane, their membrane may need to contract in order to allow for the secretion of their contents. This process has been shown in CD8 T cells to involve acid sphingomyelinase (ASMase), an enzyme which alters the composition of the membrane, forming ceramide, which curves the membrane to cause contraction [42]. Although it is not yet clear whether ASMase is important in NK cells, it is likely that a similar mechanism occurs to direct NK cell granule exocytosis. Once the lytic granules have contracted to release their contents, perforin, and granzymes work together to affect the lysis of target cells (reviewed extensively in [43]). This process occurs in and is likely facilitated by a cleft formed between the NK cell and target cell, which likely serves the function of focusing these elements to the targeted cell [22]. Following the secretion of granule contents, granule membrane proteins are internalized in a recycling process, allowing NK cells to create new lytic granules for recharging the cytotoxic capacity for serial killing of targets [41].

Conclusion

NK cell cytotoxicity represents an important line of defense against infected and transformed cells, but this ability to kill other cells is, by necessity, tightly regulated. Further studies of the

series of steps involved in the cytotoxic process will be instrumental to our understanding of how NK cells are able to kill their targets. The relationship of lytic granules with the MTOC, their ability to navigate F-actin clearances once they reach the IS, and the very nature of the clearances that allow for secretion of granule contents remain important questions. Understanding these steps that directly lead up to the directed secretion of lytic granule contents will allow for the possibility of restraining or augmenting NK cell function.

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Fig. 1.

Possible mechanisms of myosin IIA association with NK cell lytic granules. **a** Myosin IIA could attach as an individual heavy chain dimer to the granule membrane via its cargo domain, thereby using its motor domain to pull along the granule. This, however, is unlikely given myosin II structure. **b** Alternatively, myosin IIA filaments could encompass lytic granules, facilitating cooperative motor function to guide the granules along F-actin to the plasma membrane for secretion