

## REVIEW

# Cell biological steps and checkpoints in accessing NK cell cytotoxicity

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**Natural killer (NK) cell-mediated cytotoxicity is governed by the formation of a lytic immune synapse in discrete regulated steps, which give rise to an extensive array of cellular checkpoints in accessing NK cell-mediated cytolytic defense. Appropriate progression through these cell biological steps is critical for the directed secretion of specialized secretory lysosomes and subsequent target cell death. Here we highlight recent discoveries in the formation of the NK cell cytolytic synapse as well as the molecular steps and cell biological checkpoints required for this essential host defense process.**

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Natural killer (NK) cells are the innate immune system's cytolytic effector cells. Their function is critical to human host defense, as described by the severe viral infection and malignancy attributed to their absence or dysfunction.<sup>1</sup> NK cell cytotoxicity has long been recognized as a stepwise, highly regulated process that is requisite to exercising control over their powerful 'armed and ready' state.<sup>2,3</sup> Central to this process is the immunological synapse (IS), a finely tuned, specialized interface formed by an immune cell.<sup>4,5</sup> Originally described in the context of T-cell activation, the IS has now been described in many forms, and is critical for NK cell cytotoxicity, inhibition, regulation and co-stimulation (reviewed in Orange<sup>3</sup>, Barreira and Munz<sup>6</sup> and Huse *et al.*<sup>7</sup>). In the case of NK cell cytotoxicity, a primary purpose of the IS is to enable the secretion of preformed lytic granules, large specialized secretory lysosomes containing lytic effector molecules. As mediators primarily of innate immunity (a discussion of NK cells adaptive properties is beyond the scope of this review but is discussed in detail elsewhere<sup>8</sup>), NK cells integrate signals from germline-encoded activating and inhibitory receptors that have evolved to detect stressed, malignant or virally infected cells. Upon the detection of a susceptible target, it is eliminated swiftly through directed secretion of lytic granules. This requires the coordinated regulation of cytoskeletal elements, signaling molecules, cellular organelles and the lytic granules themselves. We have previously proposed four steps of NK cell lytic synapse formation and function.<sup>3</sup> Recent advances in technology have resulted in an increased understanding of the molecular coordination of each regulated step of NK cell cytotoxicity. Here we redefine these steps as we now see them, in three stages (recognition, effector and termination) comprising 48 steps and potential checkpoints in

accessing cytolytic function (Table 1). With a thorough understanding of this process, the understanding of NK cells in health and disease can be uniquely advanced and potentially more specifically exploited for therapeutic potential.

## STAGES OF LYTIC SYNAPSE FORMATION

### Recognition

The motility of NK cells in tissue or tumor microenvironment is likely driven by chemotactic signaling resulting in an approximation to key sites of interest, although NK cell motility in tumors is also driven by the expression of NKG2D ligands.<sup>9–11</sup> Therefore, similar to a T-cell sampling dendritic cells, NK cells undergo brief, exploratory interactions with multiple cell types, which may represent their initial encounter with a target<sup>10</sup> (Figure 1a). It is not known what molecules specifically mediate these interactions, although there are a number of candidate receptors expressed on resting or primed NK cells, including a variety of 'tethering' receptors as well as potentially more robust adhesion receptors. The former potentially include CD62L and PSGL-1; the latter include CD2, DNAM-1, NKG2D, the natural cytotoxicity receptors and lymphocyte function associated antigen-1 (LFA-1). Unlike T cells, a subset (~10%) of freshly isolated human NK cells express the activated conformation of LFA-1 at rest, which presumably allows adhesion to a target before the engagement of other activating receptors.<sup>12</sup> The transmission of a second activating signal through a receptor such as NKG2D, DNAM-1, 2B4 (CD244) or CD2 strengthens these interactions by further activating LFA-1, resulting in firm adhesion to the target and potentially providing minimal requirements for progression to mediating cytotoxicity.<sup>12</sup>

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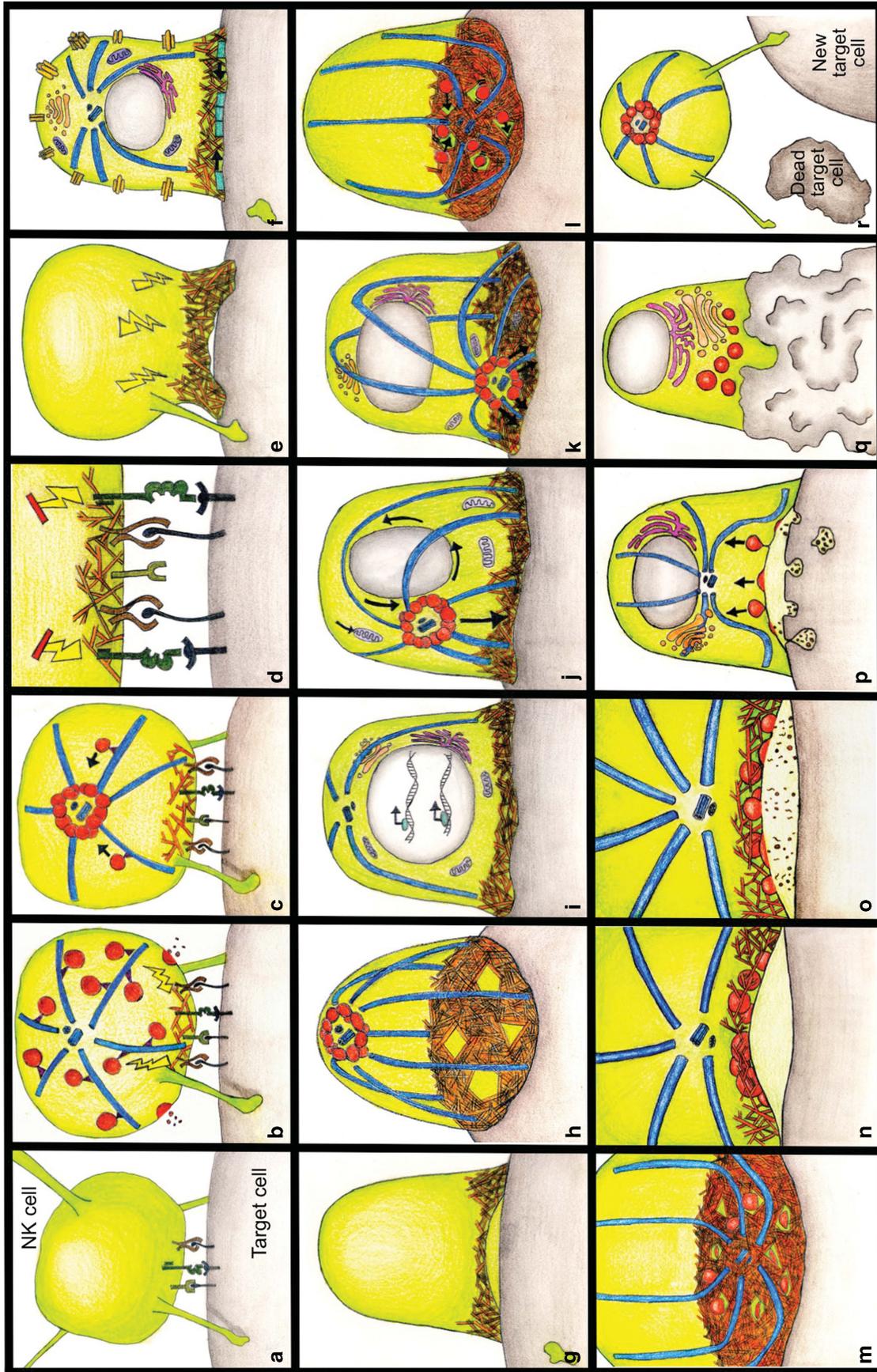
**Table 1** Stages and steps of NK cell cytotoxicity

Stage	Step	Description	Recent references	Model image (Figure 1)
Recognition	1	Approximation	9–11	a
	2	Cell tethering		
	3	Adhesion	12	
	4	Nanotube formation	13,14	
	5	Integrin-mediated signaling and arrest	18–22	b
	6	Degranulation of pre-docked granules	26,27,29	
	7	Dynein motor activation	30–32	
	8	Minus-ended lytic granule movement	30,31	c
	9	Inhibitory signaling and potentially cell release	24,25,33	d
Effector	10	Actin polymerization	47–54	e
	11	Firm adhesion	12	
	12	Actin force generation	55,56	
	13	Cell shape change	2,46,57,58	
	14	Microcluster formation	66–69	f
	15	Lipid raft coalescence	73	
	16	Sustained activating receptor signaling	Reviewed in 74,75	
	17	Actin-dependent activation signaling	Reviewed in 74,75	
	18	Ion channel activation	81,83	
	19	Lytic cleft formation	84	g
	20	Membrane transfer	85–88	
	21	Granule conduit/hypodensity formation	90–92	h
	22	Transcription factor activation	100	i
	23	Gene transcription	100	
	24	Protein synthesis		
	25	Microtubule insertion	107,113	j
	26	MTOC polarization	54,76,104–107,110,111,112	
	27	Nuclear reorientation		
	28	Golgi polarization	115	
	29	Mitochondria reorientation	118	
	30	MTOC anchoring		k
	31	Plus-ended lytic granule movement?	119	
	32	Lytic granule diffusion/motility	92,122	l
33	Hypodensity identification			
34	Lytic granule transit through cortex (hypodensities)		m	
35	Lytic granule docking	123,126	n	
36	Lytic granule priming	74		
37	Lytic granule fusion	128–131	o	
38	Externalization of granule contents	124,133	p	
39	Force generation?	122		
40	Persistence of degranulations	122		
41	Lytic granule endocytosis	28,137		
Termination	42	Relative inactivity		q
	43	Granule biogenesis	26	
	44	Downmodulation	144,145	
	45	Detachment	146	r
	46	Serial killing	147	
	47	Exhaustion (granule and energy depletion)		
	48	Recycling	28,137	

Abbreviation: MTOC, microtubule-organizing center.

Three main stages (recognition, effector and termination) are shown with discrete steps and recent, primarily NK cell-specific references from the text. Model images refer to those shown in Figure 1.

**Figure 1** Stepwise molecular progression through NK cell cytotoxicity. NK cell cytotoxicity can be broken down into three main stages: recognition (a–d), effector (d–p) and termination (q, r). During the recognition stage, the NK cell is particularly sensitive to inhibitory signaling (d). Key events include lytic granule convergence (c), the actin-dependent firm adhesion of the NK cell (e) and subsequent F-actin conduit formation (h), MTOC polarization (j) and LG fusion and exocytosis (o). This is followed by vesicle recycling (p), a period of relative inactivity (q) and detachment and subsequent serial killing (r). See text for further details.



Symbol Legend

- Nanotube
- Triggering receptor
- Inhibitory receptor
- Adhesion receptor
- Activation signal
- Inhibitory signal
- Microtubule
- Ligands
- Lytic granule
- Lytic granule contents
- Dynein/dynactin
- Filamentous actin
- Golgi network
- Endoplasmic reticulum
- Mitochondria
- Microcluster
- Ion channels
- DNA + transcription factor
- Nucleus

We are still learning of surprising interactions that NK cells engage in, likely in part due to the complex microenvironment that they patrol. One such unconventional form of communication is the formation of membrane nanotubes (Figure 1a). Nanotubes appear to serve as intercellular tethers formed rapidly after contact with the target cell and can help guide the NK cell to the target cell or provide traction during its initial interaction.<sup>13</sup> Rather than hollow tubes that mediate free diffusion, NK cell nanotubes are heterogeneous structures that transmit signals over long distances and can even deliver lytic granules from an NK cell to a target.<sup>13</sup> Importantly, their formation enhances cytotoxicity.<sup>13,14</sup> NKG2D and downstream activation molecules including DAP10 and Vav1 accumulate at the nanotube junction, resulting in the description of this junction as a nanoscale synapse.<sup>13</sup> However, blocking either LFA-1 or 2B4 does not reduce the frequency of nanotube formation, suggesting that this is not simply a stretched conventional lytic synapse.<sup>13</sup> CD2 expression specifically increases the frequency of NK target cell nanotubes, suggesting a role for specific receptor–ligand interactions in their formation<sup>14</sup> and partially explaining the observation that CD2 enhances NK cell cytotoxicity.<sup>15</sup> The mechanism by which this occurs, and the prevalence of nanotubes during routine physiological NK surveillance, remains unclear.

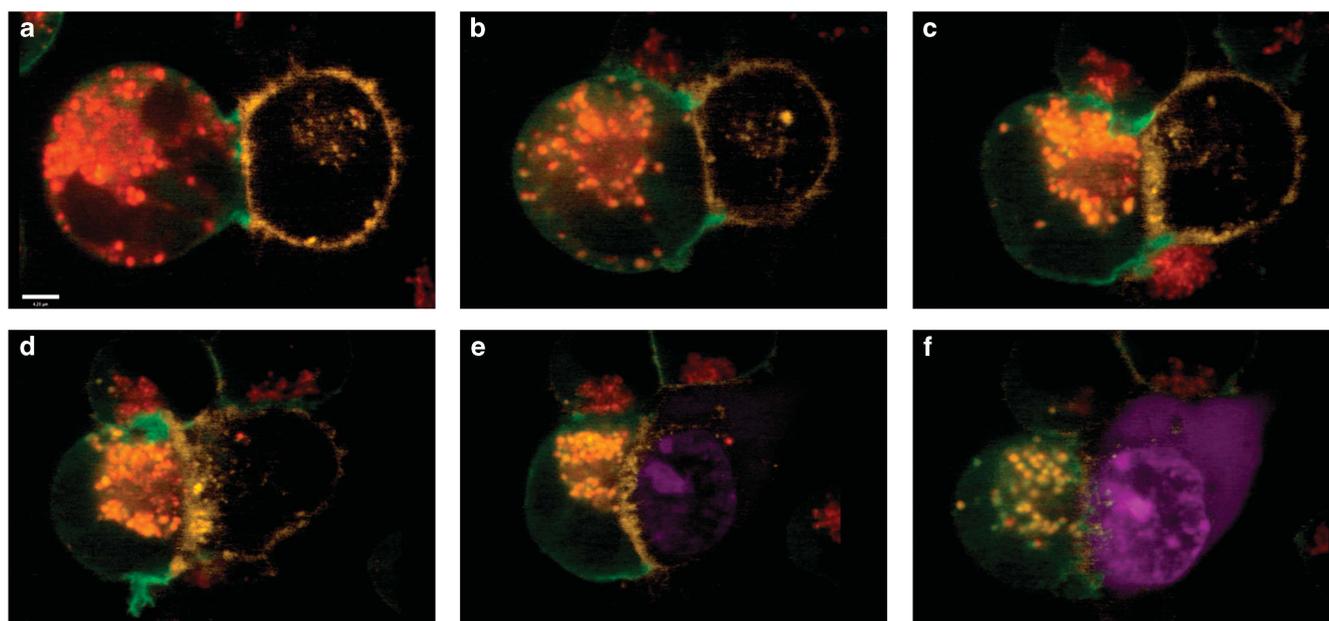
Another example of the complex initial interaction may be the newly observed phenomenon of NK cell-derived exosomes: NK surface marker-expressing, FasL and perforin-containing nanovesicles (50–100 nm) that, upon release from NK cells, can mediate target cell killing independent of IS formation.<sup>16</sup> They may also potentially transfer key ligands to target cells to enable more robust interactions in environments in which exosome concentrations are high. This concept, in concert with nanotubes, harkens to a provocative early proposal of ‘harpooning’ as a contributor to NK cell cytolytic function.<sup>17</sup> Despite their apparent constitutive secretion by human

NK cells isolated from peripheral blood, the relative contribution of exosomes to an NK-mediated cytotoxic response is poorly understood but compelling.

Following target cell contact, LFA-1 engagement initializes the first steps of IS formation, including protein tyrosine kinase activation, PIP(4,5)P2 generation and F-actin reorganization (Figure 1b).<sup>18–21</sup> In synergy with activating receptor ligation, LFA-1 also induces an arrest signal to migrating cells, with a strong activating signal resulting in a stable radially symmetrical synapse and inhibitory signaling resulting in asymmetry and subsequent resumption of migration.<sup>22</sup> Whether NK cells kill through ‘kinapses’, or moving synapses, as has been described in T cells, remains to be determined.<sup>23</sup>

LFA-1-mediated outside-in signaling for F-actin polymerization brings together all the requisite components for F-actin polymerization and initiates this process.<sup>19,20</sup> This initial signaling also includes the phosphorylation of Vav1 by a Src family kinase, its recruitment to lipid rafts and subsequent further activation as discussed in subsequent steps below.<sup>21</sup> Therefore, initial engagement of LFA-1 in concert with a second signal can help to ready an NK cell for target cell lysis. At this point the NK cell is likely still highly sensitive to a balance of activating and inhibitory signaling, and control of Vav1 phosphorylation and subsequent actin accumulation is thought to be a critical axis in the regulation of cytotoxicity.<sup>24,25</sup> At this stage, microclusters are likely present, may be functional and are also described further below.

Despite the tightly regulated steps leading to NK cell cytotoxicity, there is evidence that pre-docked granules are present in the cell cortex and can be accessed rapidly by NK cells.<sup>26</sup> Lytic granule secretion at the IS before microtubule-organizing center (MTOC) polarization has been described in primary human cytotoxic T lymphocytes (CTLs).<sup>27</sup> Although it is conceivable that antigen specificity of T cells allows for the circumvention of further checkpoints to cytotoxicity, lytic granules



**Figure 2** Stepwise stages during NK cell-mediated cytotoxicity: YTS GFP-actin (green) cells were labeled with LysoTracker Red (red) to selectively label lytic granules and co-incubated with CellMask-labeled (Life Technologies, Carlsbad, CA, USA) 721.221 target cells in the presence of SYTOX Blue (Life Technologies) (blue) for detection of cell death. Conjugates were imaged by spinning disk confocal microscopy for a total of 90 min. The images shown are frames acquired approximately every 15 min. (a) The NK cell is conjugated to the target cell and lytic granules are dispersed. (b) Granules are converged at the MTOC. (c) The MTOC along with converged lytic granules are polarized at the immune synapse. F-actin is evidently reorganized from a to c. (d) More F-actin reorganization is observed and lytic granules are docked at the plasma membrane. (e) Target cell death begins as SYTOX Blue enters the cell. (f) Target cell undergoes apoptosis and NK prepares for detachment as shown by reversal of shape change. Scale bar = 4  $\mu$ m.

are present in the cortex of resting NK cells and initial exocytosis bursts are detected (Figure 1b).<sup>26,28,29</sup> Despite these observations, imaging of both NK cell lines and primary NK cells by both live and fixed cell microscopy indicates that the majority of NK cells go through the multistep, highly regulated steps to cytotoxicity (Figure 2).<sup>2</sup> Therefore, it is unclear as to what the significance of the presence and potential exocytosis of pre-docked granules is. In this light, it is also still unclear how many lytic granules are required to kill specific types of target cells.

Polarization of the MTOC and lytic granules to the IS is required for NK cell cytotoxicity and occurs in the effector stage. However, preceding MTOC polarization, lytic granules rapidly move along microtubules and converge upon the MTOC (Figure 1c).<sup>30,31</sup> This minus-ended lytic granule movement depends on dynein motor function but is independent of actin polymerization or microtubule dynamics. It also occurs whether the NK cell is conjugated to a susceptible or resistant target cell, as it is observed in the presence of an inhibitory synapse.<sup>30</sup> This suggests that granule convergence is a step that precedes NK cell commitment to cytotoxicity, instead preparing the cell for it. Accordingly, ligation of CD28 or LFA-1 alone is sufficient to induce lytic granule convergence, as is activation by soluble interleukin-2.<sup>30</sup> Disruption of LAMP-1 (CD107a) expression also results in impaired lytic granule motility, which corresponds with a decrease in association of the dynein component p150<sup>glued</sup> with lytic granules.<sup>32</sup> Although this suggests that LAMP-1 itself may mediate the interaction of granules with dynein, the mechanism of this is unknown. Interestingly, MTOC polarization in LAMP-1-deficient cells is intact, suggesting that convergence is not a prerequisite for polarization.<sup>32</sup>

Lytic granule convergence before MTOC polarization likely represents the final juncture at which NK cells are susceptible to inhibitory signaling before a commitment to cytotoxicity. Signaling through immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing inhibitory receptors induces formation of the inhibitory synapse, which is functionally dominated by phosphatases, particularly SHP-1, leading to exclusion of the downstream signaling molecules from the contact region (Figure 1d).<sup>33,34</sup> Inhibitory signaling acts swiftly to disrupt multiple points of activation, including conjugate formation and F-actin accumulation,<sup>34</sup> LFA-1 activation,<sup>12</sup> activating receptor clustering at the IS<sup>35</sup> and critically, Ca<sup>2+</sup> flux.<sup>36,37</sup> Dephosphorylation of the activating phospho-tyrosine residues of Vav1 appears to be a critical molecular switch for this control but this response is likely fine-tuned by other factors.<sup>25</sup> These include the adaptor protein Crk, which is also phosphorylated following inhibitory signaling and recruited to the inhibitory synapse.<sup>35,38</sup> Crk, through its association with C3G and subsequent control of Rap1, likely has a role in the regulation of LFA-1 activation and subsequent target cell adhesion.<sup>38–42</sup> Interestingly, inhibitory receptors also serve to 'license' NK cells during their developmental process, and NK cells that lack the recognition of MHC molecules during maturation are rendered hyporesponsive, at least partially by reduced signaling from activating receptors to LFA-1. Unlicensed cells form fewer conjugates, but those that do conjugate mediate lytic granule polarization normally.<sup>43</sup> Other mechanisms by which these 'unlicensed' or 'anergic' NK cells are restrained in their cytotoxic capacity represents an unknown cellular checkpoint that will be of great value in understanding full access to lytic capacity.

### Effector

At the beginning of the effector stage, the NK cell commits to cytotoxicity and proceeds toward degranulation. This stage is marked largely by significant cytoskeletal rearrangements, beginning with

significant *de novo* F-actin polymerization and reorganization (Figure 1e). Although the importance of the F-actin cytoskeleton in NK cell cytotoxicity has been appreciated for some time, recently many new contributors to the regulation of F-actin have been highlighted. One of these is the Wiskott-Aldrich syndrome protein (WASp) homolog WAVE2.<sup>44</sup> Interestingly, while both WASp and WAVE2 are expressed in T and NK cells, WAVE2 may be critical for F-actin polymerization in CTLs but is normally not accessed by NK cells.<sup>45</sup> In NK cells, WASp appears to be the predominantly utilized family member. As an illustration, Wiskott-Aldrich syndrome patients who are deficient in WASp have severe NK cell functional impairment linked to an inability to rearrange F-actin.<sup>46</sup> This can be overcome with interleukin-2 treatment, which activates WAVE2 in NK cells and restores F-actin rearrangement in WAS patient NK cells *in vitro* and *in vivo*.<sup>47</sup> This not only offers therapeutic options for treatment of WAS but highlights an important difference between NK- and T-cell cytotoxicity. It also illustrates the potential cooperativity between adaptive and innate immunity via the ability of T cell-produced interleukin-2 to access distinct cell biology in NK cells.

The Rho GTPase Cdc42 is also critical for F-actin polymerization by Arp2/3 through direct binding and activation of WASp. Recently, a role for the Cdc42 effector DOCK8 has been described in NK cells.<sup>48–50</sup> Patients deficient in DOCK8 have decreased NK cell function with accompanying lytic synapse assembly and function defects that we now recognize as hallmarks of defective actin polymerization, including decreased LFA-1 recruitment, loss of granule polarization and accompanying decrease in cytotoxic function.<sup>48,50</sup> Importantly, DOCK8 deficiency does not affect overall NK cell F-actin content, but more specifically the targeted accumulation and polarization of F-actin at the synapse. This is in contrast to WASp deficiency in which NK cells have a reduced total F-actin content in addition to an inability to target actin accumulation.<sup>46</sup> Loss of DOCK2 function in mouse NK cells also results in impaired Rac activation and subsequent loss of NK cell cytotoxic function,<sup>51</sup> suggesting this family is an important regulator of NK cell cytotoxicity.

In addition to the WASp and WAVE/Scar family members, the Arp2/3 complex can be activated by cortactin.<sup>52</sup> The hematopoietic-specific homolog of cortactin, HSI, is localized to the NK cell lytic synapse, where it is critical for signaling to and from LFA-1.<sup>53</sup> In NK cells, the formin homolog hDia is not required for lytic synapse branched actin, but instead is required for NK cell cytotoxicity by targeting microtubules to the synapse and subsequent lytic granule polarization, suggesting its function as the poorly understood link between the microtubule and actin cytoskeleton.<sup>54</sup>

Once F-actin polymerization has been initiated, firm adhesion to the target cell is achieved and sustained (Figure 1e). As F-actin polymerization is required for LFA-1 recruitment and maintenance at the synapse, this synergy between actin and integrins provides firm adhesion throughout the period of NK cell activation. This transmembrane linkage results in the generation of significant force, which has been measured directly through the application of single-cell force spectroscopy. Engagement of 2B4 leads to rapid, LFA-1- and actin-dependent increase in the force sufficient to disengage an NK cell from a susceptible target.<sup>55</sup> Therefore, the mechanical forces generated at the synapse also have an important role in adhesion activation signaling. This observation is supported by the increased LFA-1-mediated adhesion of NK cells bound to targets in which intercellular adhesion molecule-1 (ICAM-1) is tethered and restrained, as opposed to freely diffusible.<sup>56</sup>

Firm adhesion to the target cell and F-actin polymerization leads to the NK cell flattening and extending the diameter of the synapse

(Figure 1f).<sup>2,46,57,58</sup> This 'cell shape change' is an actin-dependent process, as NK cells treated with actin inhibitors or from WAS patients fail to show this characteristic shape change.<sup>46</sup> Similarly, NK cells engaging in inhibitory synapses remain rounder, further underscoring the regulation of F-actin as a mechanism of NK cell inhibition.

Engagement of activating receptors leads to dynamic microcluster formation (Figure 1f). These can be defined as a critical mass of receptors that in concert can promote signal transduction. In T cells, F-actin-dependent microcluster movement occurs toward an actin-poor sink, with productive signaling occurring in the periphery of the synapse and terminating in the center.<sup>59–64</sup> Myosin IIA also is required for centripetal microcluster movement and this sustained retrograde flow of microclusters is required for sustained  $\text{Ca}^{2+}$  release and activation signaling.<sup>64,65</sup> Although the kinetics may be different in NK cells, microclusters of both activating and inhibitory receptors are present and appear to have similar dynamics compared with those in T cells.<sup>66–69</sup> Studies using NK target cell conjugates show that phosphorylated KIR2DL1-containing clusters originate in the periphery and coalesce in the center, thus reaffirming both the presence of microclusters and suggesting their utility in NK cells.<sup>67,69</sup> Furthermore, highly motile NKG2D-DAP10-containing microclusters are also seen in the periphery of the synapse, and contain high levels of phospho-tyrosine, suggesting that, as in T cells, the periphery may also be the predominant site of activating signaling in NK cells.<sup>66</sup> 2B4-containing microclusters are found centrally, suggesting segregation of activating receptors.<sup>28</sup> Interestingly, KIR2DL1 microclusters are found on resting NK cells, but become smaller and denser when NKG2D is ligated.<sup>66,68</sup> This surprising observation was made possible using super-resolution microscopy, which allows for measurement of microcluster density and size (9–20 proteins/cluster and 120 nm, respectively). It is unclear what the significance of this microcluster compression actually is, but may reflect an interplay required for NK cell licensing, as speculated previously.<sup>68,70</sup>

The lipid microenvironment may also have a role in the spatial organization of receptors (Figure 1f). The role of higher-ordered microdomains, or lipid rafts, has been somewhat contentious. The use of multivalent labels, such as cholera toxin, always raises the possibility that aggregation of these probes may result in inaccurate detection. Early studies, however, have implicated the recruitment of activating receptors to, and exclusion of inhibitory receptors from, lipid rafts.<sup>71,72</sup> Recent experiments in live Jurkat T cells using a novel phase-sensitive membrane dye and high-resolution imaging have resolved higher-order membrane at the periphery of the synapse, suggesting that these domains may support microcluster or signaling platform formation.<sup>73</sup> It remains to be seen whether this is also the case with NK cells, and what the effect of inhibitory signaling is on the membrane order at the synapse.

Activating signaling leading to NK cell activation has been extensively reviewed recently elsewhere.<sup>74,75</sup> Two main axes of signaling are initiated and sustained through cytotoxicity once appropriate receptor–ligand pairs are in place, namely, the PI3K and phospholipase  $\text{C}\gamma$  pathways.<sup>76–79</sup> Engagement of activating receptors leads to signaling through ITAM and YINM motifs and recruitment and activation of both membrane-proximal and distal proteins signaling and adaptor proteins. It is worth emphasizing the interdependence of activating signaling with the cytoskeleton, as disruption of one affects the other and prevents functional cytotoxicity. That said, the exact cellular mechanism of signaling protein localization and re-localization in NK cells remains unknown.

One key outcome of activating receptor signaling is mobilization of ions, particularly calcium (Figure 1f). Initial calcium flux occurs as a

result of rapid release from endoplasmic reticulum (ER) calcium stores mediated by IP3 channels downstream of  $\text{PLC}\gamma$  signaling.<sup>80</sup> Depletion of ER  $\text{Ca}^{2+}$  stores is sensed first by STIM1 on the ER surface. Following activation, STIM1 complexes reposition proximal to the plasma membrane (visualized as puncta on the ER surface) and activate plasma membrane-associated ORA1.<sup>80</sup> Human genetic immunodeficiency has defined critical roles for both ORA1 and STIM1 in NK cell cytotoxicity. One manifestation of these patients' disease is profound susceptibility to viral infection, a hallmark of reduced NK cell function, although they also present with many other signs and symptoms and have substantive defects in T-cell and B-cell function.<sup>1,81</sup> Importantly, while ORA1-deficient NK cells form conjugates with target cells and polarize the MTOC and lytic granules, they fail to degranulate.<sup>81</sup> Similarly, NK cells from STIM1-deficient patients fail to mediate cytotoxicity.<sup>81</sup>

Although the two  $\text{Ca}^{2+}$  flux systems are the best characterized, there are other ion channels expressed and utilized by NK cells. Recently, a critical role for intracellular  $\text{Mg}^{2+}$  has been described by a rare primary immune deficiency affecting the  $\text{Mg}^{2+}$  transporter MAGT1.<sup>82,83</sup> In these patients, intracellular levels of free  $\text{Mg}^{2+}$  are decreased, accompanied by defective NK cell and CTL killing and subsequent uncontrolled Epstein–Barr virus infection.<sup>83</sup> Interestingly, the role of  $\text{Mg}^{2+}$  in both CTL and NK cell control of Epstein–Barr virus is through maintaining the stability of NKG2D at the cell surface, suggesting an unappreciated role for NKG2D in controlling Epstein–Barr virus infection. In addition, potassium and zinc channels are expressed and utilized by T cells (reviewed in Feske *et al.*<sup>80</sup>), prompting the question of whether NK cells utilize similar mechanisms.

Presumably, in and around these steps a lytic cleft forms (Figure 1g). Once thought to be simply a gasket to prevent the leakage of lytic granules to surrounding tissues, it is now appreciated that the cleft is dynamic and complex. Much of the activity that takes place in the cleft is critical to cytotoxic function, yet remains mysterious because of its inaccessibility. Measurement of the lytic cleft by transmission electron microscopy in inhibitory and activating synapses shows that both have a similar range of distances between the NK cell and the apposing target cell membrane (10–30 nm), which are proposed to account for distribution of proteins of different sizes at the synapse.<sup>84</sup>

Electron micrographs of the NK cell synapse also show significant membrane ruffling and protrusions. These synaptic membrane features may also be a source of membrane transfer (trogonocytosis) between an NK cell and a target (Figure 1g), which has been defined for a number of receptors.<sup>85–88</sup> This process is reciprocal, as transfer of both ligand from the target cell and receptor from the NK cell is observed. Additional transmission electron microscopy experiments demonstrate that the target cell-derived receptors enter the NK cells in small, enclosed pits.<sup>88</sup> Both inhibitory and activating receptor exchange occurs across the NK cell synapse, although there is controversy as to whether cognate ligand engagement is required.<sup>85,88</sup> The functional outcome of trogonocytosis and intercellular receptor transfer is unclear, although transfer of NKG2D and MICB at the synapse results in lower NK cell cytotoxic function.<sup>87</sup> Fascinatingly, NK cells that acquire NKG2D ligand from target cells become targets themselves, and are victims of fratricide by neighboring NK cells.<sup>86</sup> This suggests that this ligand transfer may act to both enable and even control NK cell activity during infection, again suggesting the theory of harpooning.<sup>17</sup>

Following firm adhesion and the formation of a cleft, F-actin continues to undergo substantial remodeling at the synapse. Traditionally, this remodeling was thought to result in a large, central

clearing of F-actin to enable centrosomal docking and lytic granule secretion.<sup>58,89</sup> Recent advances in imaging technology, namely, the adoption of super-resolution microscopy, have revealed that ligation of activating receptors creates granule-sized conduits through which granules are secreted (Figure 1h).<sup>90–92</sup> Interestingly, viral influenza particles can also result in conduit formation, presumably through the ligation of NKp46, but this signal requires co-stimulation through LFA-1.<sup>93</sup> Other activating receptors, such as NKG2D or CD16, can signal solely for clearance formation. Although not entirely understood, this seems to be an elegant mechanism by which an NK cell could discern a free viral particle from one bound to a target cell. Whether proteins on the granules themselves form lytic granule conduits, they are a result of the inherent dynamism at the synapse, or occur in specific hotspots of signaling remains to be determined. It seems likely, based on the presence of actin nucleating factors at the synapse, that F-actin is dynamic and is likely undergoing continual remodeling. This may contribute to conduit kinetics, as has been suggested by analyses of synaptic plane actin dynamics using total internal reflection fluorescence imaging.<sup>92</sup>

The process of remodeling and specifically breaking down as opposed to building is of particular interest as conduits in F-actin are a requirement for degranulation.<sup>91,92</sup> Cofilin, which severs and depolymerizes F-actin, works in concert with Arp2/3 to remodel actin to enable secretion in muscle cells.<sup>94</sup> Coronin 1A, which promotes the turnover of actin through binding to both Arp2/3 and cofilin, regulates Arp2/3 localization to the T-cell synapse, although a role for this important immune cell regulator in NK cells has yet to be defined.<sup>95,96</sup> One appealing hypothesis is that hypodensity formation occurs at regions of high activating receptor density as a result of highly localized signaling events, such as is seen in mast cells.<sup>97</sup> However, this is difficult to reconcile with what is likely the dynamic movement of microclusters of activating receptors themselves. An alternative explanation is that lytic granules create their own conduits, enabled by the presence of actin-remodeling proteins on their surface. This would be compatible with the observation that Myosin IIA is present on lytic granules and appears required for their penetration through F-actin,<sup>98,99</sup> but this hypothesis has not been directly tested.

Perhaps, in preparation for potential serial killing or NK cell recycling, lytic synapse formation also activates transcription factors and gene transcription (Figure 1i). Ligation of NKp30 results in rapid translocation of nuclear factor  $\kappa$ B to the nucleus and *de novo* protein synthesis.<sup>100</sup> Perhaps, not surprisingly, perforin and granzyme is refilled in CTL lytic granules while they kill.<sup>101,102</sup> The newly produced perforin then reaches the synapse independently of conventional lysosomal granules and mediates cytotoxicity.<sup>103</sup> Whether NK cells employ such a mechanism remains to be determined, as does the precise program of gene transcription that is activated during cytotoxicity.

Following the dynamic rearrangement of the actin cytoskeleton, microtubule dynamics result in a dramatic reorientation of the MTOC and associated lytic granules toward the synapse (Figure 1j). Requirements for centrosome polarization include LFA-1, Pyk2, ERK2, CIP4, the formin hDia and Vav1.<sup>54,76,104–107</sup> However, it is important to note that F-actin polymerization is required for MTOC polarization,<sup>46,53,54,58,108,109</sup> therefore, any interference with F-actin dynamics will subsequently impair MTOC and granule polarization.

One consideration in MTOC polarization is the significant amount of force likely needed to generate this reorientation. It is assumed from studies in other systems that microtubule insertion and anchoring in the cell cortex lead to either pushing (microtubule

growth) or pulling (microtubule shrinkage) forces that can reposition the centrosome. Dynein may again have a role, as a minus-ended motor it can generate significant pulling forces on shrinking microtubules when anchored in the cortex, and may contribute to the fine-tuning and positioning of microtubule asters.<sup>110</sup> Accordingly, it was recently shown that in T cells, MTOC repositioning occurs as a result of end-on capture shrinkage of microtubule focused at the center of the IS and anchored to cortical dynein.<sup>111</sup> Interestingly, in NK cells, it appears that kinesin-1 has a role in the initial movement of the MTOC to the synapse, mediated through interactions with the small GTPase Arl8b.<sup>112</sup> IQ motif containing GTPase-activating protein 1 (IQGAP1) may act as a linker between CLIP-170 on the plus ends of microtubule and specific regions of cortical actin. Loss of IQGAP1 results in a failure of NK cells to polarize the MTOC and degranulate.<sup>113</sup> Cip4 has also been implicated as a link between microtubules and F-actin at the cortex.<sup>107</sup> Although in T cells the MTOC docks in contact with the plasma membrane at the synapse, this has not been directly observed in NK cells.<sup>54,114</sup>

As the MTOC polarizes to the synapse, cellular organelles also reposition with some moving toward and others away from the synapse (Figure 1j). Reorientation of the Golgi along with microtubules toward the IS presumably aids in directed secretion of granules toward the target cell.<sup>115</sup> In T-helper cells, the mitochondria polarize toward the synapse to maintain  $\text{Ca}^{2+}$  flux across the plasma membrane for T-cell activation.<sup>116,117</sup> In NK cells, the mitochondria reposition toward the NK cell IS following NK stimulation with anti-NKGD2 antibodies but not with anti-KIR2DL1 antibodies, suggesting that the mitochondrial dynamics are triggered as a result of NK cell activation.<sup>118</sup> The polarization of these organelles is important for sufficient  $\text{Ca}^{2+}$  influx for signaling and granule exocytosis. It is conceivable that polarized mitochondria further serve as local sources of energy to power synaptic function, although this needs to be proven.

Following MTOC polarization to the IS and anchoring at the plasma membrane, the delivery of the polarized lytic granules to the synapse occurs (Figure 1k). In T cells, this process requires plus-ended, kinesin-1-dependent movement of lytic granules upon microtubules to the membrane.<sup>119</sup> In NK cells, a role for kinesin-1 in this process has not been described but is conceivable. Other cellular machinery could also have a role in lytic granule movement. As mentioned above, Myosin IIA is associated with lytic granules and required for NK cell cytotoxicity.<sup>98,99,120</sup> Whether this is through the facilitation of short runs across F-actin following MTOC polarization, the penetration of granules through the actin meshwork or the exocytosis of lytic granules<sup>121</sup> is unclear, although obviously these scenarios are not mutually exclusive.

Following delivery to the plasma membrane, lytic granules have significant dynamic movement, which is followed by arrest, and subsequent degranulation in only a subset of granules (Figure 1l).<sup>92,122</sup> This movement is not dependent upon F-actin dynamic rearrangement,<sup>122</sup> but a role for microtubule dynamics has not been excluded. Granule movement may be undirected rolling on synaptic actin, seeking regions of hypodensity. Alternatively, it may represent the requirement for granules to find a primed fusion/tethering complex to mediate their arrest, as is suggested by high-resolution imaging of lytic granules in CTL utilizing Munc 13-4 Rab27a complexes for granule arrest and subsequent degranulation.<sup>123</sup>

At some point, lytic granules must traverse the pervasive actin network at the synapse (Figure 1m). Lytic granule may mechanically identify areas of hypodense F-actin regions to pass through,

or directed F-actin remodeling at the activating synapse may be required for clearing the way for lytic granules to transit through minimally permissive F-actin hypodensities. However, even in the presence of F-actin clearances, it is likely that lytic granule transit through the cortex requires the generation of force as was suggested by experiments evaluating degranulation in the absence of an F-actin meshwork.<sup>122</sup> Myosin IIA may mechanically facilitate lytic granule motility through the F-actin-rich area of the IS or may be involved in inducing local changes in the F-actin structure to allow polarized lytic granules access to the plasma membrane.<sup>74,98,99</sup>

After polarizing to the IS and before degranulation, lytic granules dock at the IS and fuse with the plasma membrane (Figure 1n).<sup>26,120,124,125</sup> Docking is likely mediated primarily by Munc 13-4 and Rab27a, both of which are recruited to lytic granules following activating receptor ligation.<sup>126</sup> In CTL, lytic granules become fully mature only at late stages of the exocytic pathway, when Munc13-4 and Rab27a associate with perforin- and granzyme-containing lysosomal components.<sup>127</sup> Upon interacting with Rab27a, Munc13-4 may also regulate the interaction between vesicle and target soluble NSF Attachment Protein REceptor (SNARE) required for lytic granule fusion with the plasma membrane.<sup>123</sup> Alternatively, this may be mediated by the interaction of Syntaxin 11 and Munc18-2 with R-SNAREs (such as VAMP7) on the lytic granule membrane.<sup>74</sup> Priming then occurs as a result of Munc13-4-mediated activation of Syntaxin 11 or bridging of the plasma membrane with lytic granule. Fusion occurs as a result of SNARE complex formation, and although this complex has not been defined in NK cells, mutations in the SNARE components Syntaxin 11, VAMP4 and VAMP7 prevent NK cell degranulation.<sup>128–131</sup>

In mast cells, coordinated oscillations of calcium in concert with N-WASP and PIP(4,5)P2 dynamics trigger granule fusion with the plasma membrane.<sup>97</sup> Whether such localized signaling dynamics contribute to NK cell exocytosis remains to be seen, although this hypothesis would complement the localized generation of actin hypodensities. Vesicular exocytosis is also triggered by calcium flux. In addition to the global calcium reserves such as the ER and the store-operated reservoirs, local  $\text{Ca}^{2+}$  nanodomains created by channels on the lytic granule membrane in T cells are essential for their exocytosis.<sup>132</sup> Thus, local synaptic membrane nanoalterations may factor into a final step in allowing the release of lytic granule contents.

Granule exocytosis may be an additional mechanism of regulating cytotoxicity (Figure 1o). NK cell lytic granules use at least two distinct modes of fusion: (1) complete fusion where granule content is completely discharged and the contents diffuse rapidly at the plasma membrane and (2) incomplete fusion, whereby formation of a transient fusion pore at the plasma membrane is accompanied by release of some but retention of most of the granule contents.<sup>124,133</sup> This latter form of degranulation is suggestive of alternate forms of granule fusion seen in other secretory cells such as chromaffin cells and neurons. These are descriptively named 'kiss and run',<sup>134</sup> in which a granule pore is formed and resealed, preventing full release of granule contents, 'kiss and stay',<sup>135</sup> in which granules remain at the membrane and are re-acidified, presumably for rapid recycling or reuse and 'crash fusion', in which granule contents are released without stable docking and priming.<sup>136</sup> The true existence and significance of these alternate forms of granule fusion in NK cells is incompletely understood, although it is conceivable that it could be a means of facilitating serial killing through the rapid recycling of granule contents. As such, two-way vesicular trafficking occurs at the NK cell synapse, and rapid Munc13-4-clathrin mediated endocytosis of granule membrane proteins exposed at the plasma membrane is

necessary for the recycling of a pool of endocytic vesicles, allowing the serial killing event (Figure 1p).<sup>28,137</sup> F-actin dynamics may have a role in the extrusion of granule contents and the persistence of lytic granules at the membrane following exocytosis.<sup>122</sup> Although this is presumably owing to force generation, the direct proof is presently unavailable.

After degranulation, a period of relative inactivity begins and is presumably required as lytic granule contents cross the lytic cleft to act upon the target cell (Figures 1o and p). The sustained presence of fused granules at the synaptic membrane, however, may serve additional functions as well. Interestingly, the exposure of LAMP-1 on the NK cell surface protects the NK cell from cytotoxicity-induced suicide,<sup>138</sup> although this is likely not the only mechanism.<sup>139–141</sup> Subsequently, uptake of lytic granule contents by the target cell initiates apoptosis and marks the end of the effector stage of cytotoxicity, as the lytic hit has been administered.

### Termination

The termination stage of the synapse refers to the steps that immediately follow the release of lytic granules and up to either the reinitiating of a subsequent recognition or return to a resting state. Initially, there is a longer time of apparent relative inactivity. During this time the target cell continues its death process, primarily by apoptosis (Figure 1q). Perforin induces membrane flipping on the target cell, resulting in exposure of phosphatidylserine.<sup>142</sup> Phosphatidylserine on the surface of the cell may provide signaling to the NK cell to terminate the response, as NK cells express proteins that have been shown to bind to phosphatidylserine such as the ITIM-containing molecule CD300a.<sup>143</sup> A further mechanism by which the NK cell could relax its activation status is through receptor downmodulation as has been documented for CD16 and NKG2D.<sup>144,145</sup> Perhaps, this could apply to other activating receptors and cell adhesion molecules.<sup>3</sup> Ongoing is new LG biogenesis during this relatively quiet period as a likely requirement for NK cell 'recharging' and subsequent serial kills.<sup>26</sup>

The final stage of cytotoxicity is defined by detachment from the target cell, a process that is not entirely arbitrary. Kinetic studies show that the decision to end stable contacts with target cells is made faster when killing occurs.<sup>146</sup> Interestingly, detachment was sometimes seen along with the formation of nanotubes connecting the NK and target cells (Figure 1r). The purpose of these connections in the context of a dying cell, however, is unclear and they may just represent the default behavior of an NK cell to tether to activating objects of interest.

Insight into the steps of the termination stage can be seen during the process of NK cell serial killing. Serial killing can demonstrate burst kinetics, with a delayed first kill followed by more rapid subsequent kills.<sup>147</sup> A proposed mechanism for burst kinetics is the idea of kinetic priming, which suggests that NK target interactions depend upon recent killing events. It is proposed that kinetic priming is a continuity of signaling, as interactions with old targets remain until a new target is found.<sup>147</sup> As such, lytic granule may remain converged to the MTOC following target cell lysis in preparation for subsequent kills, as previously proposed.<sup>30,31</sup>

A consequence of serial killing is a near depletion of lytic granules and cytotoxic effector molecules. This depletion can leave the NK cells in an exhausted state until they detach from the target cell that has depleted their contents. After separation, exposure to locally available activating factors such as interleukin-2 can restore their cytotoxic function.<sup>148</sup> New granule biogenesis and new effector molecule synthesis presumably mediate this restoration. Interestingly, in  $\text{CD8}^+$  cytotoxic T cells, it was found that rapid upregulation of

nongranule-restricted perforin can be transported to the IS and participate in cytotoxicity.<sup>103</sup> As this form of perforin was identified in NK cells,<sup>103</sup> perhaps this observation carries over to NK cells and contributes to the reacquisition of effector function after exhaustion. Although seemingly the least is known about the specifics of the termination phase of the synapse, it can be interpreted as one of the most important parts, as recycling of NK cells *in vivo* in diseased tissue environments is likely to be of the essence in host defense.

### Summary and future thoughts

In recent years, explosive advances in technology have driven us deeper within the NK cell to better understand the mechanism of its critical function. In doing so, we have a much better understanding of the tightly regulated steps leading to cytotoxicity. There are critical and fundamental questions that remain to be answered, some of which require substantive technology development. These include, but are not limited to the following: (1) the exact number of, and temporospatial requirements for, degranulation events needed to kill particular target cells; (2) how both inhibitory signaling and 'anergy' restrain progression through the cell biological steps of synaptic formation; (3) the mechanism by which conduits in the cell cortex are created to enable lytic granules to reach the NK cell synaptic membrane; (4) the true purpose of lytic granule convergence to the MTOC, (5) the mechanism of delivery of lytic granules from MTOC to plasma membrane; and (6) what specifically governs the release of an NK cell from its target cell. In addition, we still have much to learn about the fine-tuning of the cytotoxic response and the subversion of it by viruses and malignant cells. These represent only a sampling of the many questions begotten by the last 15 years of discovery, the answers to which will provide the tools to manipulate and control cytolytic host defense. Better understanding of these facets of NK cells will enable more effective harnessing of NK cell functions and therapeutic intervention.

### ACKNOWLEDGEMENTS

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