Natural killer cell granules converge to avoid collateral damage

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To clear infection, cytotoxic lymphocytes must destroy target cells while avoiding nonspecific killing of surrounding healthy cells. In this issue, Hsu et al. (2016. *J. Cell Biol.* https://doi.org/10.1083/jcb.201604136) use live-cell imaging to show that lytic granule convergence protects bystander cells from unintended death by promoting polarized secretion of soluble cytolytic proteins toward the intended target.

Cytotoxic lymphocytes, including natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), possess the unique capacity to instruct infected or malfunctioning cells to undergo regulated apoptosis. This mechanism is essential for clearing the body of virus-infected and tumorigenic cells. Cytolytic attack is triggered through specific receptor–ligand interactions between the target cell and the immune cell. All cytotoxic lymphocytes secrete a variety of death-inducing proteins including perforin and granzymes that act together to induce apoptosis of the target. These cytotoxic proteins are housed in modified lysosomes called lytic granules, which must fuse with the plasma membrane to release their payloads. Target cell death then relies on the diffusion of the secreted cytolytic proteins through the extracellular medium to reach their target. Although this strategy allows cytotoxic lymphocytes to destroy practically any cellular target, it presents a problem in the context of an organism. Infected or tumor cells often reside in tissues where they are surrounded by healthy "bystander" cells, creating the potential for significant collateral damage.

How do CTLs and NK cells avoid nonspecific cellular destruction when secreting cytolytic factors? Immunofluorescence studies of conjugates formed between cytotoxic lymphocytes and target cells long ago revealed that the microtubule organizing center (MTOC) polarizes to the contact interface with the target, known as the immunological synapse (Kupfer et al., 1983). Lytic granules converge at the MTOC through the action of dynein, and the resulting bolus of granules is delivered to the synapse en masse (Stinchcombe et al., 2006; Mentlik et al., 2010). Granule convergence in CTLs has been shown to occur rapidly after first contact with the target but before MTOC polarization to the synapse, which takes ∼6 min (Ritter et al., 2015). This mechanism of targeted granule delivery facilitates directional secretion specifically toward the target (Fig. 1). Directional secretion could theoretically limit off-target bystander killing by confining soluble lytic proteins between the immune cell and target, but this has never been directly shown.

In this issue, Hsu et al. provide convincing evidence that granule convergence in NK cell–mediated killing leads to polarized, unidirectional secretion, which enhances the efficiency of target killing and also protects bystander cells from an untimely death. To make these observations, the authors developed an imaging strategy to monitor lysis of target or bystander cells after either polarized or nondirectional secretion of lytic granules. To trigger nondirectional versus directional secretion, the authors leveraged a previously developed strategy using *Drosophila melanogaster* S2 cells as inert surrogate targets onto which various stimulatory signals can be presented (Bryceson et al., 2005). Opsonizing S2 cells with Ig antibodies triggers signaling through NK cell CD16, an IgG Fc receptor, which leads to nonpolarized lytic granule secretion. Overexpression of intercellular adhesion molecule 1 (ICAM-1) on the surface of S2 cells stimulates signaling through LFA-1 of the NK cells, which leads to granule convergence but not secretion. Combining these signals by opsonizing ICAM-1–expressing S2 cells with IgG led to granule convergence and unidirectional secretion toward the target.

To test the differential effects of directional versus nondirectional secretion in target killing, Hsu et al. (2016) used a live-cell imaging strategy combining 4D confocal and an ultrasound-guided acoustic-trap microscopy system, which allows the user to control the location of cells on a coverslip (Christakou et al., 2015). With this strategy, the authors could coordinate the position of NK cells and their targets to determine the precise moment of cell–cell contact. Imaging lytic granules with lysotracker, Hsu et al. (2016) observed granule convergence upon NK cell interaction with Ig-coated S2 targets expressing ICAM-1. Cell death was measured by uptake of SYT OX blue viability dye, which only labels cells with a compromised plasma membrane. NK cells interacting with Ig-coated ICAM-I–negative S2 cells did not converge granules and were less efficient in killing their targets.

The strategy of coordinated positioning of cells combined with live imaging and readouts of cytotoxicity allowed the authors to design experiments to answer the difficult question of how directional secretion might reduce collateral damage in tissues. To do this, the ultrasound-guided acoustic-trap microscopy system was used to arrange clusters of inert bystander cells around activating S2 cells that would stimulate either directional or nondirectional secretion. Hsu et al. (2016) then monitored cell death in the nonactivating bystanders to measure "collateral

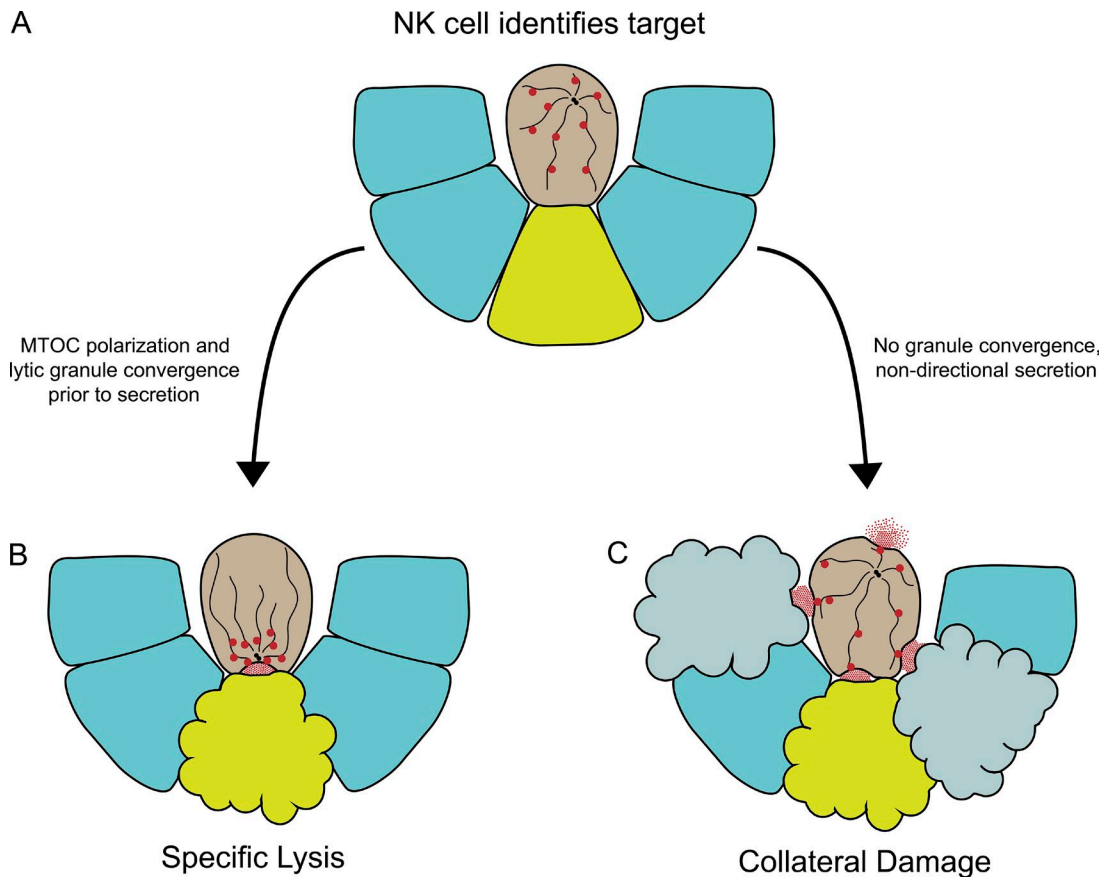Correspondence to Alex T. Ritter: ritter.alex@gene.com

Figure 1. **Directional secretion of lytic granules could serve to protect healthy tissue.** (A) NK cells (brown) may identify targets (yellow) that reside in tissues surrounded by healthy bystander cells (teal). (B) Convergence of lytic granules (red) and polarization of the MTOC to the target cell interface encourages directional secretion of granules toward the target. Concentration of secreted cytolytic proteins in the synaptic cleft enhances target killing efficiency and prevents destruction of bystander cells. (C) Lack of a granule convergence signal leads to nondirectional secretion and possible collateral damage to tissue.

damage." Activation conditions that triggered nondirectional NK cell secretion in these simulated tissues resulted in a greater degree of bystander death around the activated NK cell. The idea that directed secretion serves to decrease nonspecific killing has been a presumption in the literature, but this is the first time that it has been directly shown. To reinforce this observation, Hsu et al. (2016) induced nondirectional degranulation in clusters of human target cells using two different strategies. Granule convergence is dependent on the motor protein dynein. Treatment of NK cells with the small molecule dynein inhibitor ciliobrevin D before engagement of target cells decreased granule convergence and led to nondirectional secretion. Blocking the interaction of LFA-I on the NK cell with ICAM-I on the target with an antibody also induced nondirectional secretion. In both of these cases, nondirectional secretion increased the level of collateral damage observed in bystander cells.

Hsu et al. (2016) have performed well-designed experiments to dissect the role of granule convergence along the microtubule cytoskeleton in directing polarized secretion in NK cells. This opens up some intriguing new questions about how other factors might play a part in regulating secretion that occurs outside of the immunological synapse. A growing body of evidence suggests that the actin cortex plays an important role in regulating the secretion of lytic granules at the synapse in both CTLs and NKs. The actin cortex is a dense mesh of interwoven actin filaments, myosin motors, and actin binding proteins that abuts the interior of the plasma membrane in animal cells. It

has been proposed to act as a physical barrier to secretion, preventing large secretory vesicles from coming close enough to the plasma membrane to dock and fuse in unstimulated cells. In CTLs, the actin cortex has been shown to undergo a dramatic reduction in density, specifically at the synapse within 60 s of target encounter (Ritter et al., 2015). The decrease in actin density at the center of the synapse precedes the arrival of converged lytic granules with the MTOC, suggesting its importance in facilitating granule secretion. Two studies have used superresolution microscopy to get a detailed look at the actin cortex of NK cells in the context of lytic granule secretion (Brown et al., 2011; Rak et al., 2011). In these studies, NK cells were plated on glass coverslips coated with activating ligands or nonactivating ligands, fixed after a period of time, and stained for actin and other markers. Both groups demonstrate that the density of the actin cortex at the interface of NK cells on an activating surface is reduced compared with NK cells plated on a nonactivating surface. Lytic granules preferentially localize (Brown et al., 2011) and secrete (Rak et al., 2011) at areas of actin "hypodensity" on the activating interface, suggesting that some coordination of granule localization and actin density is important for secretion. Brown et al. (2011) showed that CD16 stimulation by itself, which leads to nonpolarized secretion, results in cortical actin remodeling at the synapse that produces regions of actin hypodensity large enough for lytic granule secretion. The focus on actin reorganization in these NK cell studies is restricted to the cell membrane touching the glass.

Examination of the actin cortex in the rear of the cell is difficult in these superresolution microscopy studies, but it raises the question as to what kind of changes in cortical actin density, if any, may be happening outside the synapse to facilitate nondirectional granule secretion.

Hsu et al. (2016) show that in NK cells, nondirectional secretion results in a lower probability of specific lysis of the intended target. Recently, Kabanova et al. (2016) reported that B cells are able to evade the cytolytic attack of CTLs by inducing nondirectional lytic granule secretion. The nondirectional secretion resulting from CTL interaction with B cells is remarkably similar to that described by Hsu et al. (2016) in NKs. It remains to be seen whether nondirectional secretion induced by immune response to B cell malignancies results in tissue damage in the B cell follicle, bone marrow, or vasulature where B cells may reside. The experiments described by Kabanova et al. (2016) implicate ADP ribosylation factor–like protein 8, a kinesin-binding adapter protein, in driving lytic granules to the periphery of CTLs after engagement with B cell targets. The signals on B cells that drive nondirectional secretion in CTLs have yet to be described, but it is possible that these could be used by other tumors to escape CTL-based cytolysis. Thus, the techniques developed by Hsu et al. (2016) could be useful in fleshing out mechanisms of immune suppression in CTLs and NK cells alike.

In conclusion, as CTL and NK cell activity continues to be leveraged in immunotherapeutic treatments of cancer, understanding the details of how these cells specifically destroy their targets is becoming more important. Identifying and controlling the signals that induce directional and nondirectional lytic granule secretion could lead to new ways to use these cells as weapons against cancer.

Acknowledgments

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