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Anatomy of a murder: how cytotoxic T cells and NK cells are activated, develop, and eliminate their targets

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Granule-mediated cytolysis is undoubtedly the most important effector function of $CD8^+$ T cells and natural killer (NK) cells. After a killer cell recognizes its target, the cytotoxic granules, which are specialized secretory lysosomes that store the death-inducing proteins, migrate to the immunological synapse (IS). The cytotoxic granule membranes then fuse with the killer cell plasma membrane to release their contents to induce programmed death of the cell targeted for destruction. The cytotoxic granules store the death-inducing proteins in a way that protects the killer cell from auto-destruction, so that they are available for rapid mobilization when a target to be eliminated is recognized. The principal death effectors are the serine proteases [called granzymes (Gzm), for granule enzyme] and the membrane perturbing proteins, perforin (PFN) and granulysin. Although killer cells can also destroy their targets by engaging death receptors such as Fas, studies using PFN knockout mice have clearly shown the importance of the granule exocytosis pathway for controlling viral infection and tumors. The same basic mechanisms are used by all killer cells, whether they are $CD4^+$ or $CD8^+$ cytotoxic T lymphocytes (CTLs) or NK cells. Individual killer cells express distinct subsets of cytotoxic mediators, but they are only expressed after activation. It is now 7 years since the last comprehensive review of killer cells and the mechanisms by which they eliminate their targets (1). It is time for an update.

This volume of *Immunological Reviews* dissects recent progress in understanding the anatomy of the murder perpetrated by killer cells. We also review recent advances in understanding how the expression of the effector molecules involved in cytolysis and the activation and differentiation of killer cells and memory cells, which can be rapidly mobilized to combat

infection, is regulated. In the past few years, research has also begun to focus on other functions of the death-inducing enzymes, both in targeting viruses and inducing inflammation. Recently, there has been an explosion of research in innate immunity, which is the first line of defense against pathogens, which also shapes the later adaptive immune response. At the same time that investigators have uncovered the sensors and signaling pathways by which pathogens and other forms of danger are sensed by the immune system, research on NK cells, the innate killer cells, has improved our understanding of how NK cells distinguish 'self' from 'non-self' cells that need to be eliminated. In the process, many of our old ideas about NK cells have needed to be revised.

Familial hemophagocytic lymphohistiocytosis (FHL) is a clinical syndrome caused by defects in killer cell granule exocytosis. The article by Schmid *et al.* (2) reviews what has been learned from studying this syndrome. Recent studies that identified the genes mutated in FHL, when combined with previous mutations associated with more generalized defects in the trafficking of secretory lysosomes, have been key to beginning to understand how cytotoxic granules dock at the plasma membrane and release their contents into the IS formed with a target cell destined for elimination. This has turned out to be a complicated process. FHL patients and PFN knockout mice have difficulty coping with viral infection. The mice are also tumor-prone, but the human patients in the past died so early in life that they did not develop cancer. The most striking clinical manifestation of FHL is the uncontrolled activation of T lymphocytes and macrophages. Activated macrophages engulf red blood cells, which is the origin of the syndrome's name. It is likely that much of the T-cell and macrophage activation in FHL is triggered by viral infections that do not resolve because of the underlying defect in cytotoxicity. The review discusses intriguing recent results in mice that suggest that blocking interferon-γ (IFN-γ), which is secreted in response to infection and which is a potent macrophage activator, can both prevent and treat the salient disease manifestations due to uncontrolled macrophage activation. This approach might be attractive for treating the symptoms of FHL patients, although blocking IFN-γ could interfere with the antiviral immune response. The ultimate treatment to address the underlying disease is bone marrow transplantation.

Dustin and Long (3) review recent progress in dissecting the events that occur at the IS formed by the killer cell with its targets. Both NK cells and killer T cells organize their regions of interaction with their targets into a bull's eye pattern. In the center is the central supramolecular activation cluster (cSMAC), divided into a signaling domain containing signaling molecules [which might include the receptors (T-cell receptor, NK activating and inhibitory receptors, FcR-γ) and their associated adapters and kinases] and an effector domain with docking sites for cytotoxic granule release. The cSMAC is surrounded by a peripheral ring (pSMAC) of adhesion molecules and a third distal ring (dSMAC) composed of cytoskeletal components. In the past 5 years, the focus has been on how activating and inhibitory signaling is integrated at the synapse in NK cells and on the kinetics and stability of IS formation. The most effective killer cells (i.e. $CD8^+$ T cells) appear to form a more stable and symmetrical synapse, while less effective $CD4^+$ T-cell killers form an asymmetric, potentially less well sealed and more transitory synapse (kinapse). One important question, which is still unresolved, is whether the IS forms a tight gasket that segregates cytolytic effector molecules from the extracellular milieu. One intriguing possibility suggested by the loose imperfect rings of the kinapse is that these structures might be more likely to leak their contents, which could potentially cause bystander cell damage and inflammation.

The next group of reviews focuses on the cytolytic effector molecules. PFN is an essential molecule for granule-mediated cell death, because it delivers the death-inducing Gzms into the target cell. There is only one delivery molecule and many Gzms. Recent elegant structural studies coupled with analysis of PFN mutations and their clinical consequences in FHL patients have begun to elucidate a picture of how this pore-forming protein multimerize in membranes in a calcium-dependent manner to form pores. These studies are reviewed by Veskoboinik *et al.* (4). There remains a good deal of controversy about how PFN actually delivers the Gzms into the host cytosol. The simplest and original model is that PFN forms pores in the cell membrane through which Gzms enter the cell. However, the data do not really support this model. If Gzms entered through membrane pores, one would expect the Gzms to diffuse into the cytosol. However, they are first found in endosomes and only then released into the cytosol. A revisionist theory, put forth initially by Froelich (5), posited that PFN acts to release Gzms from endosomes. Our data actually suggest that PFN probably acts at both membranes – it first forms pores in the cell membrane that lead to an influx of extracellular calcium that immediately triggers a membrane repair response. The repair response activates rapid endocytosis to remove damaged membrane (including the PFN pores themselves). In a study published after these articles were written (6), we have recently been able to visualize PFN for the first time in target cells. Both PFN and Gzms colocalize

in endosomes and then Gzms are released after about 10–15 min into the target cell cytosol. How the Gzms escape from endosomes requires further study, but the most likely scenario is via PFN pores in the endosomal membrane.

PFN expression is tightly regulated. Unlike the Gzms, some of which can be expressed in non-cytolytic cells, PFN, which is absolutely required for cytotoxicity, is exclusively expressed only by killer cells. Pipkin *et al.* (7) review their elegant studies defining the *cis*-regulatory regions of the Prf1 gene responsible for this tight control and discuss what is known about the transcription factors that regulate Prf1 expression.

One of the issues that researchers have been grappling with recently is why there are so many Gzms (five in humans and 10 in mice). The Gzms are differentially expressed in different types of cytolytic cells, and individual killer cells express their own unique complement of killer molecules. Not much is known about how the expression of each of the Gzms is regulated, both about the activation signals that differentially induce their expression and about how long expression is sustained as killer cells develop into different types of effector and memory cells. Understanding in detail the regulation of Prf1 and Gzm gene expression should be a fruitful area of research in the next few years. An overview of the Gzms is provided by Anthony *et al.* (8). Gzms A and B are the most widely expressed Gzms, expressed in all types of killer cells. GzmM is more widely expressed in NK cells and may have a more important role in innate immunity. The other Gzms ('orphan' Gzms) are less abundant and not too much is known about them. GzmA and GzmB activate distinct pathways of programmed cell death, and it is likely that the other Gzms may also activate still other death programs that remain to be described. Although most immune studies of killer cells have focused on GzmB, GzmA$^{-/-}$ and GzmB$^{-/-}$ CTLs are equally potent at killing target cells, and mice deficient in either Gzm are mostly similarly immunocompetent, suggesting that *in vivo* both Gzms are similarly potent as death inducers (9). The Gzms have distinct proteolytic activities, and each enzyme appears to cleave a small number of distinct substrates with a lot of specificity. GzmA activates caspase-independent cell death, via unique mitochondrial and DNA damage pathways that have recently been described and are reviewed by me (9). A recent study suggests that this pathway may also be activated by an endogenous protease during some forms of neuronal cell death. GzmB activates the caspase apoptotic pathway both by initiating cleavage of effector caspases and by directly cleaving key caspase substrates, such as bid and ICAD, as reviewed by Afonina *et al.* (10). It has become clear recently that some substrates are different for mouse and human GzmB. Recent studies, reviewed by Bovenschen and Kummer (11), have also begun to identify substrates of the orphan Gzms. The most likely reason for the redundancy of the Gzms is that some tumors and viruses have evolved ways of evading one pathway or the other. For instance, overexpression of bcl-2 or other anti-apoptotic bcl-2 family members, other inhibitors of apoptosis, or viral serpins protects cells from GzmB but not other Gzms. Another reason for having multiple Gzms is to facilitate elimination of common viruses, as discussed in the review by Andrade (12). An elegant example of interplay of GzmH and GzmB in defense against adenovirus infection was first described by Andrade. The distinct duplications of Gzm genes in mice and humans might reflect the coevolution of each host with its own unique set of pathogens.

One of the themes of several of the reviews is emerging evidence that the Gzms also are involved in non-cytolytic activities, principally activating inflammation (8–10, 12). The Gzms have been shown to degrade extracellular matrix proteins, which could play a role in allowing CTLs access to sites of infection or could be pro-inflammatory. GzmB is widely expressed without PFN in myeloid cells, suggesting a non-cytolytic role. Moreover, in states of chronic infection and inflammation, GzmA and GzmB are both detected in serum and other body fluids. The recent picture of the asymmetric IS (the kinapse) suggests a mechanism for Gzm leakage, especially from CD4^{+} T cells or possibly in other situations where stimulation by the target cell is not extremely strong. The early literature on Gzms showed that GzmA cleaves and activates interleukin-1β (IL-1β) (13), which we have verified, and a number of studies suggested other potential pro-inflammatory roles of GzmA. However, the pendulum has swung to such an extent that some researchers think that the Gzms, especially GzmA, may play a more important role in activating inflammation than in cytotoxicity (14). In that study, the concentration of GzmA required to activate macrophage cytokine secretion was much lower than the concentration needed to induce cell death. However, those data need to be considered in relation to the micromolar concentration of Gzms estimated to be present in the IS versus the picomolar concentrations in body fluids normally and nanomolar concentrations present in the most extreme states of chronic inflammation. Moreover, new mechanisms by which the Gzms activate inflammation have not yet been described, and *in vivo* evidence is still sparse. Moreover, the GzmA used in the recent study that argued for the relative importance of GzmA in activating inflammatory cytokines was not rigorously shown to be free of endotoxin and had reduced cytolytic activity relative to

other preparations. I suspect that the Gzms will turn out to be important modulators of inflammation in the setting of chronic infection, but that their cytolytic and antiviral activity is actually their main function. In the coming years, *in vivo* experimental data should help resolve this controversy.

To round out the set of articles on Gzms, Ashton-Rickardt (15) reviews the role of endogenous Gzm inhibitors (serpins) in protecting killer cells from their own weapons of destruction and in memory cell development. Serpins or other extracellular serine protease inhibitor or small molecule Gzm inhibitor-type drugs might be useful therapy to prevent the inflammatory effects of the Gzms. The review of Kurschus and Jenne (16) discusses the possibility and difficulties in developing cytolytic drugs based on GzmB.

Cells dying by programmed cell death, but not necrotic cells, are rapidly engulfed by phagocytic cells. Engulfment reduces the inflammatory signals induced by dying cells, since inhibiting engulfment or the nucleases that degrade apoptotic DNA leads to autoimmunity and inflammation. One of the important ways that apoptotic cells are recognized is via their externalization of phosphatidyl serine (PS) to the outer leaflet of the plasma membrane. Freeman *et al.* (17) discuss the TIM family of PS receptors, one of which was originally identified as mutated in a mouse strain prone to asthma. The TIM family has recently been shown to play an important role in regulating immune tolerance in many settings via its role in clearing apoptotic cells.

Understanding the activation signals that regulate development of effector and memory cells is important for understanding immune responses to infection, immunopathology, and how to induce long-term memory for a vaccine. The next set of articles considers this question from different viewpoints. During an immune response, naive T cells differentiate into a heterogeneous mix of cells with varying effector and memory cell properties. Some of the confusing heterogeneity may result from looking at a dynamic phenotypic snapshot that is evolving over time or from restimulation of cells by an unresolved infection before the cell has come to a resting memory state. These cell fates are not fixed but can be manipulated by different strength, timing, and duration of signals from antigen, binding to costimulatory and inhibitory receptors, and inflammatory signals from triggering innate immune pathogen recognition or danger sensors present on the killer cell itself, antigen-presenting cells, $CD4^+$ T-helper cells, or other nearby cells. The activating signals can be cell-associated or provided by cytokines. This area of research is reviewed by Arens and Schoenberger (18). LeFrancois and Obar (19) discuss the differentiation of naive $CD8^+$ T cells to early effector cells, short-lived effector cells, and memory precursor cells, and the signals that regulate this process. The review by Rutishauser and Kaech (20) discusses what is known about the transcriptional regulators that govern whether an activated naive $CD8^+$ T cell becomes a short-lived effector cell or survives as a long-lived memory cell. Araki *et al.* have recently found that the immunosuppressive drug rapamycin promotes $CD8^+$ T-cell differentiation to memory cells. They discuss how the mammalian target of rapamycin (mTOR) favors both the survival of $CD8^+$ memory precursor T cells during clonal expansion and the decision to differentiate into a memory cell over a short-lived effector cell (21). This finding potentially has important implications for vaccines, which aim to optimize long-term memory. It is clear that we still have a lot to learn about how extracellular signals are integrated both spatially and temporally into producing this complex mix of responding cells. How epigenetic modifications prepare a memory cell to respond quickly by expressing cytolytic and other antiviral genes is just beginning to be examined and will likely be another area of fruitful research. Moreover, it is highly likely that post-transcriptional events, particularly microRNAs, also regulate this process, but this approach has not yet been studied. Almost all of these studies have been performed in mice where it is possible to manipulate gene expression or protein function. Figuring out to what extent the mouse models being developed translate to humans is daunting, since few experimental manipulations are possible and the subjects under study are heterogeneous both genetically and in their historical exposure to infection and other environmental factors. In fact, prior exposure to other pathogens alters the response to a new infection, in part because of cross-reactivity of the T-cell receptor for antigenic peptides, as reviewed by Welsh *et al.* (22).

The last set of reviews focuses on recent developments in our understanding of NK cells. Champsaur and Lanier (23) focus on the growing family of ligands of the NK cell activating receptor NKG2D, which is also expressed by $CD8^+$ CTLs and γδ T cells. Unlike most NK receptors, which are rapidly evolving, NKG2D and some of its ligands are conserved between mice and human, suggesting that it may have an especially important role in innate immune defense. NKG2D recognizes major histocompatibility complex-like molecules that are expressed in response to stress. Modulation of NKG2D ligand expression and their shedding from the cell surface can have important consequences for tumor and viral immune protection or evasion.

One of the longstanding principles of innate immunity was the idea that innate immune responses are not altered by expo-

sure to infection or other antigen. This idea has been overturned in the past few years by studies of NK cells. As reviewed by Paust *et al.* (24) and Cooper and Yokoyama (25), prior infection or exposure to antigens of NK cells can augment their response on rechallenge. This innate immune 'memory' does not involve antigen receptor rearrangements. In the examples found so far, it is mediated by the clonal expansion of subsets of NK cells bearing NK receptors that recognize specific antigens or by the enhanced cytokine production by NK cells previously activated by soluble cytokines and by cell surface engagement with dendritic cells. The alterations in clonal proliferation, cytokine production, and tissue trafficking (and chemokine receptor expression) of experienced 'memory' NK cells upon reencounter with antigen likely reflect epigenetic reprogramming of key functional genes. I expect we will learn more about this in the next few years.

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