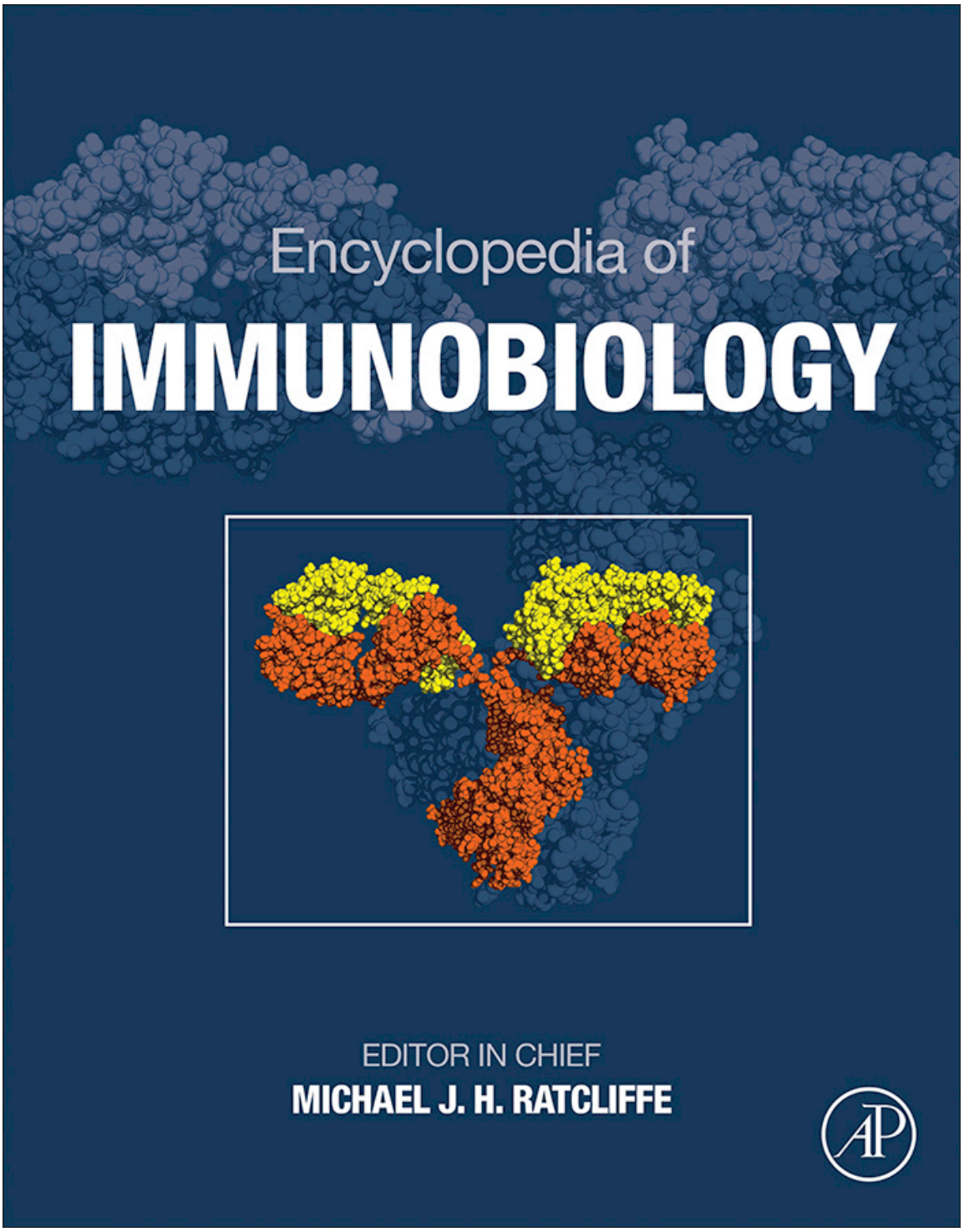

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Cytotoxic Lymphocytes

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Glossary

Adaptive immunity Also called acquired immunity, the specific response to infection or cancer that involves recognition of nonself molecules by specific receptors on B and T lymphocytes.

ALPS Autoimmune lymphoproliferative syndrome, a rare genetic syndrome caused by survival of activated lymphocytes because of defects in death receptor signaling.

Antigen A molecule that binds to immune recognition receptors on B and T lymphocytes.

Apoptosis Programmed cell death, a highly regulated cell death process that involves nuclear fragmentation, chromatin condensation, DNA damage, and membrane blebbing that is distinct from necrosis, cellular death caused by loss of cellular membrane integrity.

c-FLIP A death receptor regulator that inhibits downstream signaling and cell death.

Caspase A family of cysteine proteases that cleave substrates after aspartic acid residues and when activated cause apoptosis or pyroptosis.

CD4 Cluster of differentiation 4, a cell surface glycoprotein of the immunoglobulin superfamily, highly expressed on a subset of T lymphocytes (helper T cells) and more weakly expressed on myeloid antigen–presenting cells that bind to MHC class II molecules and serve as a coreceptor for the T cell receptor.

CD8 Cluster of differentiation 8, a cell surface glycoprotein dimer of the immunoglobulin superfamily, highly expressed on a subset of T lymphocytes (cytotoxic T cells) and more weakly on natural killer cells and some dendritic cells that bind to MHC class I molecules and serve as a coreceptor for the T cell receptor.

Cytokine A soluble small protein released by a variety of immune and nonimmune cells that bind to specific cell surface receptors to mediate complex signaling programs in responding cells.

Cytotoxic granule A specialize secretory lysosome in killer lymphocytes that contains cytotoxic effector molecules that are released when a killer cell recognizes a target cell to eliminate.

Cytotoxic T lymphocyte A T lymphocyte that has cytotoxic granules and is capable of killing target cells when its T cell receptor recognizes an antigen on the target cell.

Death receptor A TNF receptor family cell surface receptor, which forms a trimer and is activated by ligation with a counter-receptor; its ligation can trigger caspase-mediated apoptosis.

DISC Death-inducing signaling complex, a multicomponent complex that organizes around activated death receptor trimers.

DR4 Death receptor 4 or TRAIL receptor 1, a TNF family member that binds TRAIL and activates apoptosis.

DR5 Death receptor 5 or TRAIL receptor 2, a TNF family member that binds TRAIL and activates apoptosis.

FADD Fas-associated protein with death domain, an adaptor protein that bridges a death receptor to caspases 8 and 10 to form the DISC.

Familial hemophagocytic lymphohistiocytosis FHL, a rare autosomal recessive genetic syndrome that is often fatal if untreated, characterized by uncontrolled proliferation and activation of lymphocytes and macrophages caused by genetic mutants that impair granule-mediated death.

FAS A death receptor, also known as CD95, encoded by the TNF receptor superfamily member 6 gene (*TNFRSF6*), whose ligation by FAS ligand triggers caspase-mediated apoptosis.

Granule exocytosis The process by which cytotoxic granules migrate to the immune synapse, dock on the killer cell plasma membrane and fuse with it, releasing their cytotoxic effector molecule contents into the synapse.

Granulysin A small protein in the cytotoxic granules of killer cells that disrupts microbial membranes and delivers granzymes to kill them.

Granzyme A family of homologous serine proteases stored in cytotoxic granules that is delivered into target cells by perforin and triggers caspase-dependent or caspase-independent programmed cell death in cells marked for immune elimination.

IL-1 Interleukin-1, an inflammatory cytokine that causes fever.

Immune synapse The interface between an immune lymphocyte and the target cell or antigen-presenting cell it recognizes.

Innate immunity The part of the immune system that does not require prior exposure for function, distinguished from adaptive immunity.

Interferon A family of cytokines produced in response to infection or cancer that function to enhance immune responses and inhibit viral infection.

Major histocompatibility complex MHC, a set of cell surface proteins, which differ between individuals, which control T lymphocyte responses by binding to small molecule antigens; MHC molecules bound to antigen are recognized by T cell receptors, some NK receptors, CD4, and CD8; they determine histocompatibility or the ability to accept donor grafts.

Memory T cell T lymphocytes that have previously responded to antigen that persist after the initial exposure and can respond rapidly when they reencounter the same antigen.

Microtubule-organizing center A eukaryotic cell structure from which microtubules form and attach.

Mucosal-associated invariant T cell A subset of T lymphocytes which express a specific T cell receptor (Vα7.2-Jα33 in humans) that recognizes microbial byproducts.

Natural killer cell Innate cytotoxic lymphocytes that lack T cell receptors and are generated in the bone marrow.

Necroptosis A programmed form of necrosis caused by death receptor activation when the caspases are inactivated.

NKT cell A subset of T cells with a restricted set of T cell receptors that recognize lipid antigens presented by cell surface CD1d.

Perforin A pore-forming molecule in the cytotoxic granules of killer lymphocytes that multimerizes in cholesterol-containing membranes in a calcium-dependent manner and delivers the granzymes into target cells.

Phagocytosis The process by which one cell engulfs another and digests it.

Serglycin An acidic protein made up of many serine–glycine repeats that serves as a scaffold for cytolytic molecules in cytotoxic granules.

T cell receptor A dimeric immunoglobulin superfamily receptor on the surface of T lymphocytes that recognizes antigen presented by MHC molecules; its ligation signals T cell activation via CD3 molecules that associate with it.

TRADD Tumor necrosis factor receptor type 1–associated death domain protein, an adaptor that links an activated death receptor with downstream signaling molecules that signal NF-κB activation or cell death.

TRAIL TNF-related apoptosis-inducing ligand (CD253), the ligand for the DR4 and DR5 death receptors.

Tumor necrosis factor receptor TNFR, a family of cytokine receptors that form trimeric complexes on the cell surface to initiate a variety of cellular responses, including apoptosis, necroptosis, inflammation, and cell proliferation.

γδ T cell T lymphocytes, whose T cell receptor is composed of γ and δ subunits, rather than the more common αβ subunits, which concentrate in the mucosa and skin, that recognize common microbial antigens.

Abstract

Killer lymphocytes provide powerful immune protection from cancer and infection by eliminating dangerous cells. Innate killer cells (principally natural killer cells) and innate-like T cells provide the first response. Antigen-specific immunity by cytotoxic T lymphocytes takes over about a week later and provides life-long immunity. When killer cells recognize a target cell, they mobilize cytotoxic granules filled with death-inducing pore-forming proteins (perforin, granulysin) and proteases (granzymes) to cause noninflammatory programmed death of the target cell. The pore-forming proteins deliver the granzymes into target cells and microbes, where they cleave multiple proteins to activate multifocal programs of cell death. Killer cells also control the immune response to infection by eliminating activated T cells once an infection has cleared using an alternate death pathway activated by death receptors on the target cell.

Introduction

Killer cells defend against infection and cancer by destroying cells that need to be eliminated (Lieberman, 2013; Lieberman, 2010a). They survey tissues and lymphoid organs, crawling about in search of infected, malignant, or damaged cells. When their receptors identify a cell to be eliminated, they bind tightly to the target cell, forming an immune synapse (Bromley et al., 2001; Dustin and Long, 2010). Into the synapse they dump death-inducing enzymes from specialized cytoplasmic granules, called cytotoxic granules, that activate the cell death machinery present within all cells to cause the targeted cell to self-destruct by a process called apoptosis. Nearby normal cells are not harmed. The dying cell is recognized by scavenger cells, such as macrophages and dendritic cells, which engulf and degrade it in a process called phagocytosis without sounding immune alarms of danger. However, when these scavenger cells digest the dying cell, they also enhance the immune response to the infection or cancer by displaying the unique molecules present in the targeted cell, such as viral or tumor immunogenic peptides, called antigens, to other immune cells able to recognize the target cell, activating them to generating more immune cells specific for the infection or cancer. Killer cells also eliminate the immune cells that respond to an infection after the infection has been cleared (Fas et al., 2006). This quiets down the immune response. For this purpose, killer cells use a second strategy – they bear receptors that recognize 'death receptors' (DRs) on the activated immune cells that need to be eliminated. Binding to the DR on the target cell causes the targeted cell to self-destruct.

Who Are the Killers?

Killer cells can be grouped into two general types (**Figure 1**). The first group (innate lymphocytes) is first responders that recognize shared features of infected, cancerous, or stressed cells. They patrol the surfaces of the body where most infections enter, as well as the blood and internal tissues, and do not need to be activated in advance.

Natural Killer Cells (Sun and Lanier, 2011)

The best-studied innate killer cells are natural killer (NK) cells, which arise from common lymphoid progenitor cells in the bone marrow. They are important in the immediate response

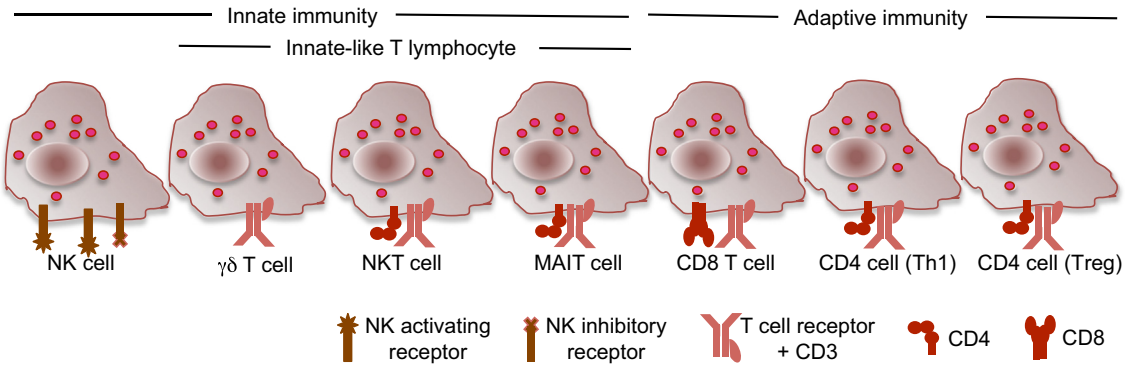

Figure 1 Killer cells. Lymphocytes with the capacity to kill can be classified as innate immune killer cells – immediate responders, which are activated by common features on infected or cancerous cells that need to be eliminated – or adaptive immune killer cells, which recognize molecules that are unique to a pathogen or cancer. The innate immune cells are present before any exposure, while the antigen-specific adaptive killer cells only develop after an individual has encountered an infection or cancer or been vaccinated. Innate killer cells include natural killer (NK) cells that do not express a T cell receptor and T lymphocytes that express invariant T cell receptors that recognize classes of infectious agents. These include γδ T cells, NKT cells that recognize glycolipids, and mucosal-associated invariant T (MAIT) cells that recognize bacterial synthesized vitamin derivatives. The adaptive immune killer cells are all T cells. The $CD8^+$ T cells are the most potent and important class of killers.

to a new infection. NK cells express multiple activating and inhibitory receptors and, whether or not they are triggered to kill depends on the balance among these competing signals. NK cell–activating receptors recognize cell surface changes in tumors, stressed cells, and infected cells, such as downregulation of major histocompatibility complex (MHC) molecules, cell surface expression of nonclassical MHC molecules induced by stress, or poorly characterized cell wall components of fungi (Li et al., 2013). Freshly minted NK cells have minimal cytotoxic activity (Fehniger et al., 2007) because they only weakly express perforin, the rate-limiting cytotoxic granule protein for killing. However, perforin and the other death-inducing enzymes and cytotoxicity are upregulated rapidly when NK cell–activating receptors are stimulated and are believed to stay up for the life of the NK cell. Previously immunologists thought that prior antigen exposure did not alter innate immune responses, that is, that there is no innate immune memory of infection. However, NK cytotoxicity to infection and other stimuli can be greatly increased by previous exposure (Paust and von Andrian, 2011; Marcus and Raulet, 2013). NK cell memory of prior exposure probably results from the expansion of NK cells bearing activating receptors specific for different pathogens. Most NK-activating receptors are poorly conserved during mammalian evolution, suggesting that they may have coevolved with important pathogens. However, the link between individual NK receptors and pathogens remains to be defined.

Cytotoxic T Lymphocytes

The other major type of killer cells are T lymphocytes, which also arise from common lymphoid progenitor cells, but develop in the thymus and express unique T cell receptors that can respond to specific peptide antigens. The most abundant and potent killer T cells are $CD8^+$, and their T cell receptors recognize eight to nine amino acid peptide antigens presented by class I MHC on the surface of target cells. However, some $CD4^+$ T cells, especially those that are generated by viral infection, which recognize peptide antigens presented by class II MHC, also have cytotoxic granules and kill. These antigen-specific CD4 and CD8 T lymphocytes are the killer cells of adaptive immunity. They do not express any of the death-inducing enzymes in their naïve state (before they have encountered the antigen their T cell receptor recognizes). After their T cell receptor is activated, the naïve cells are activated first to proliferate for about 5 days generating thousands of copies of themselves and then to become killer cells. The first phase is called clonal expansion and the second is called differentiation. It takes about a week after the beginning of an infection to generate large numbers of antigen-specific killer T cells (also called cytotoxic T cells or CTLs) (**Figure 2**). In people who have never encountered an infectious agent, in the first week, NK cells and innate-like T cells provide most immune protection.

CTL Activation

Full activation to killer cells requires that the naïve T cell is activated by three signals – (1) the antigen, which is recognized by the T cell receptor; (2) a costimulatory signal, such as CD80 or CD86, which is only expressed by dedicated activated immune antigen-presenting cells, such as dendritic cells and macrophages, which bind to costimulatory receptors on the T cell, such as CD28 or the inducible T cell co-stimulator (ICOS); and (3) an alarm signal, produced in response to a pathogen or sign of danger (Lanzavecchia and Sallusto, 2001). Typically the alarm signal will be a soluble mediator ('cytokine'), such as interleukin-1 or interferon, produced by the target cell or an antigen-presenting cell when its receptors recognize pathogen-associated or danger-associated molecules. Examples of alarm signal triggers are viral or bacterial DNA or RNA in the cytoplasm of an infected cell or bacterial cell wall or flagellar components. Because killing of normal uninfected or noncancerous cells would be dangerous and lead to autoimmunity (the immune system attacking normal tissue), this three component activation system acts as a brake. However, in some circumstances this brake (or checkpoint) gets in the way of effective protection. For example, killer cells

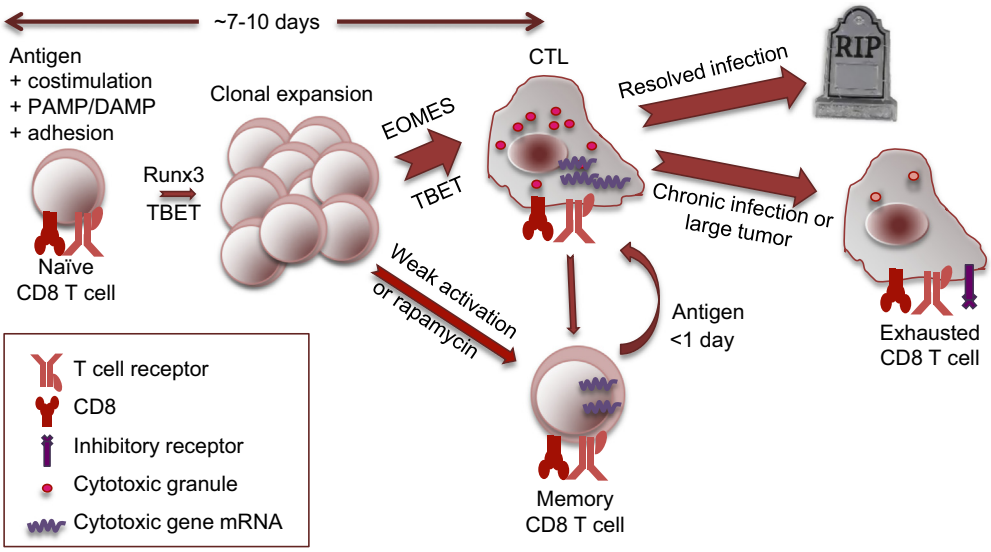

Figure 2 Generation and death of $CD8^+$ cytotoxic T lymphocytes (CTL). Naïve $CD8^+$ T cells are generated in the thymus and lack the killing machinery (cytotoxic granules) unless they are strongly stimulated by an antigen that their T cell receptor recognizes together with other stimuli that confirm that the source of the antigen is a foreign protein worth attacking. The transcription factors Runx3, TBET, and eomesodermin (EOMES) activate the transcription of genes needed for the naïve cell to proliferate and differentiate into a killer cell. After the danger has been cleared, most killer cells die, but a small fraction survives as fast-acting memory cells. If the danger persists, the activated cells survive, but lose the ability to respond and kill.

that are repeatedly activated by a tumor or infection downregulate their costimulatory receptors and instead express inhibitory receptors that cause the killer cell to lose its ability to kill – by not being fully activated and by no longer expressing death-inducing enzymes (Wherry and Kurachi, 2015). Administration of antibodies that block inhibitory coreceptors from signaling and interfere with this checkpoint are showing promising clinical benefit in some cancer patients, awakening killer cell–protective immunity and tumor immune surveillance (Topalian et al., 2015).

Memory Killer Cells

When an infection has cleared, most of the specific T cells that became CTLs die in a process called antigen-induced cell death. DRs, expressed by CTLs when they are activated to kill, are recognized by other killer T cells, which eliminate them. A small proportion of CTLs, however, survive as memory T cells. Memory T cells do not express killer enzymes or contain cytotoxic granules and hence no longer kill. However, the chromatin surrounding killer cell genes is opened up compared to that in naïve cells and poised for expression. As a consequence, memory cells, generated in response to infection or vaccination, rapidly become cytotoxic within hours, providing protection almost immediately.

Innate-like Killer T Cells

Although T cells are generally considered part of adaptive immunity, some types of T cells function like innate cells. They are called innate-like T lymphocytes, and they are especially abundant at the surfaces of the body, such as the skin and gut, where most pathogens invade the body. These innate-like killer T cells include γδ T cells, NKT cells, and mucosal-associated invariant T (MAIT) cells. Their T cell receptors have limited diversity and recognize molecules that are common to broad classes of infectious agents presented by nonclassical MHC molecules. For example, NKT cells recognize pathogen-derived lipids presented by CD1 molecules and MAIT cells recognize bacteria-derived vitamin byproducts, not synthesized by mammalian cells (Brennan et al., 2013; Corbett et al., 2014; Kjer-Nielsen et al., 2012).

Cytotoxic Granule–Mediated Death

Cytotoxic Granules

Cytotoxic granules are specialized secretory lysosomes that store killer cell molecules in an acidic environment, a condition under which they are inactive, protecting the killer cell from its own weapons of destruction (Luzio et al., 2014; Stinchcombe and Griffiths, 2007; **Figure 3**). Similar secretory organelles are found in other blood-derived cells, including platelets, mast cells and osteoclasts, endothelial cells, pigment-producing melanocytes, and some types of neurons. The killer cell enzymes, all of which are positively charged, are retained in the cytotoxic granule by binding to a negatively charged granule protein called serglycin. Cytotoxic cell granules contain two types of effector molecules – pore-forming proteins that disrupt cell membranes (perforin, granulysin) and proteases (granule enzymes or granzymes). The pore-forming proteins, which are only expressed in killer cells, deliver the granzymes into the target cell where they activate programmed cell death (Voskoboinik et al., 2010; Law et al., 2010; Thiery and Lieberman, 2014). To protect the killer cell from its own death-inducing enzymes, effector molecules are synthesized as an inactive precursor (proenzyme) that is processed to its active form only once within

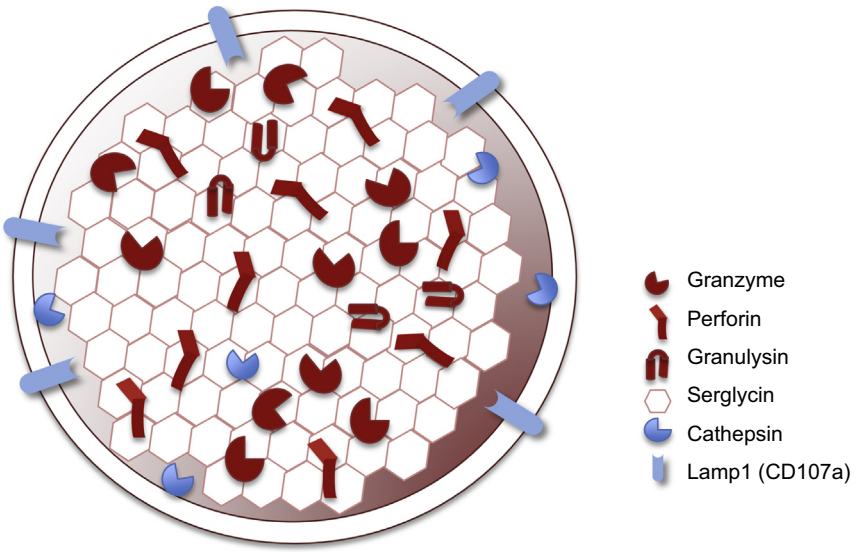

Figure 3 Cytotoxic granules. Cytotoxic granules are specialized secretory lysosomes that contain lysosomal enzymes (cathepsins) and lysosomal membrane proteins, such as LAMP1, as well as specialized cargo – the death effector proteins, granzymes, perforin, and granulysin, held in the granule by a serglycin matrix.

the cytotoxic granule. If any stray granzymes leak out of the granules, the killer cell is protected by cytosolic serpins, serine protease inhibitors that inactivate the granzymes (Kaiserman and Bird, 2010).

Granule Exocytosis (de Saint Basile et al., 2010)

When the killer cell recognizes a target cell, it forms an immune synapse with the target cell that is stabilized by a ring of adhesion molecules (Bromley et al., 2001; Dustin and Long, 2010; Figure 4). The cytotoxic granules are initially tethered to microtubules and localized near the microtubule-organizing center (MTOC), away from the synapse (Figure 5). As soon as the killer cell is activated, the MTOC and its associated cytotoxic granules quickly migrate to the immune synapse where the cytotoxic granules dock on the CTL cell membrane. The membrane of the cytotoxic granule then fuses with the CTL plasma membrane, releasing its contents into the immune synapse. The synapse is tightly sealed like a suction ring, confining the deadly killer cell enzymes to the interface with the target cell. Perforin (Figure 6) then forms small pores in the target cell membrane, which lead to endocytosis of the other cytolytic molecules into the target cell (Thiery et al., 2010; Thiery et al., 2011). Within the target cell endosome, perforin forms larger pores that deliver the granzymes into the target cell cytosol to initiate a complex series of proteolytic events that cause death of the target cell.

The killer cell is not damaged, even though its cell membrane is exposed to the same cytolytic molecules as the target cell. How the killer cell is protected is not completely understood. Perforin is the limiting factor for triggering death. One idea is that lysosomal membrane–bound proteases, displayed on the killer cell membrane after granule exocytosis, cleave and inactivate any perforin molecules redirected toward the killer cell (Balaji et al., 2002). Killer cells are serial killers (Bossi et al., 2002). Usually the encounter with a target causes release of only some of the cytotoxic granules, leaving the killer cell armed for further attacks. Once the killer cell has started to kill the target cell, it detaches and crawls away, searching for other nearby target cells. The signal that causes detachment of the killer cell is not known.

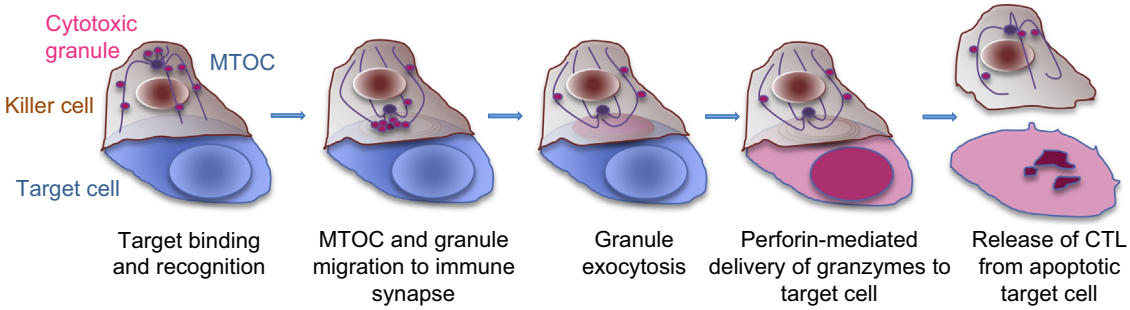

Figure 4 Granule-mediated death. When a killer cell recognizes a target cell, it forms an immune synapse and mobilizes its cytotoxic granules toward the target cell. The granules dock at the synapse and fuse their membranes with the killer cell plasma membrane to release their contents into the immune synapse. Perforin delivers the other granule proteins into the target cell to initiate its killing. The killer cell is unharmed by the encounter and crawls away to seek out additional nearby potential targets. MTOC, micotubule organizing center. Figure reprinted with permission from Lieberman, J., 2013. Cell-mediated cytotoxicity. In: Paul, W.E. (Ed.), Fundamental Immunology. Wolters Kluwer/Lippincott Williams and Wilkins, Philadelphia, pp. 891–909.

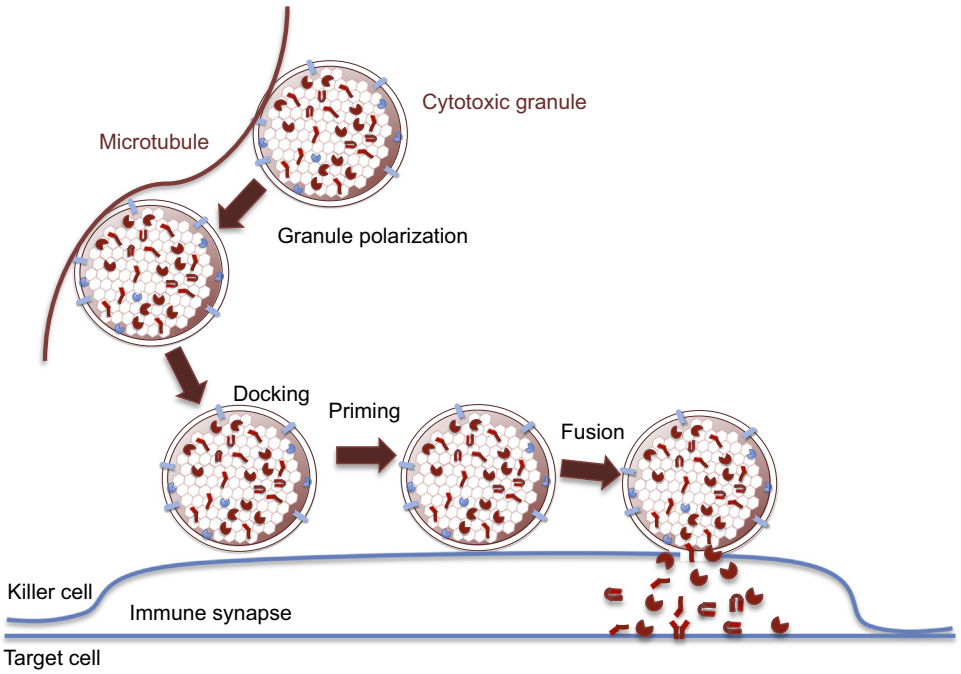

Figure 5 Steps in granule exocytosis. After a killer cell recognizes a target cell, its cytotoxic granules move along microtubules toward the immune synapse. They dock at the immune synapse and undergo a series of membrane changes that cause the granule membrane to fuse with the cell membrane, releasing their death-inducing contents into the immune synapse.

Humans with genetic defects in granule-mediated death, caused by inactivating mutations in perforin or in molecules required for granule docking and release, are profoundly immunodeficient and are especially handicapped in controlling viral infections. Children with these mutations develop familial hemophagocytic lymphohistiocytosis (FHL), a severe immune activation syndrome that is often fatal in childhood, unless treated with bone marrow transplantation (de Saint Basile et al., 2010; Stepp et al., 1999). Some patients with milder perforin mutations that do not completely eliminate cytotoxic function are not diagnosed until adulthood. These adult FHL patients not only have impaired antiviral immunity but are also prone to develop lymphoma. The most prominent and sometimes fatal clinical manifestation of FHL is due to macrophage activation and inflammation caused by uncontrolled activation and expansion of CD8 T cells, often in response to poorly controlled herpesvirus infections. The expanded population of activated CTLs secrete copious amounts of interferon-γ and other cytokines that systemically activate macrophages.

Granzyme-Mediated Death (Chowdhury and Lieberman, 2008; Lieberman, 2003)

The granzymes are proteases, structurally related to digestive enzymes like trypsin or chymotrypsin (**Figure 7**). Humans have 5 granzymes (granzymes A, B, H, K, and M) and mice have 10. Each killer cell expresses a different combination of these enzymes. Granzymes A and B are the most abundant and well studied. Not much is known about how the other 'orphan' granzymes cause death, but it is likely that each granzyme on its own can cause cell death by activating distinct death pathways. In fact, mice deficient in individual granzymes are unimpaired in immune defense or only mildly more susceptible to specific viruses. No human granzyme–deficient diseases have been described. Granzyme B activates the classical caspase-dependent apoptotic pathway used during development to eliminate unnecessary cells (Darmon et al., 1995; Andrade et al., 1998). It cleaves and activates the effector caspase, caspase 3, and also directly cleaves some of the key caspase substrates in cells, such as bid and the inhibitor of the caspase-activated DNase (ICAD), which are important in the mitochondrial and DNA damage pathways of apoptosis, respectively. Granzyme A activates a parallel caspase-independent programmed cell death pathway (Lieberman, 2010b). The multiplicity of granzymes means that it is hard for a target cell to develop resistance to killer cell attack. In fact there are no known examples of resistant cells. Although some tumor cells have developed strategies to resist death by granzyme B, they are still sensitive to the other granzymes. Once the granzymes are released into the target cell cytosol, they concentrate in the nucleus and mitochondria of the target cell (Martinvalet et al., 2008; Trapani et al., 1996). In the nucleus they preferentially cleave DNA- and RNA-binding proteins; in the mitochondrion they disrupt electron transport to generate superoxide radicals that play a critical role in executing death.

Other Roles for Granzymes?

In addition to their central role in killing, the granzymes may also act extracellularly to promote inflammation and

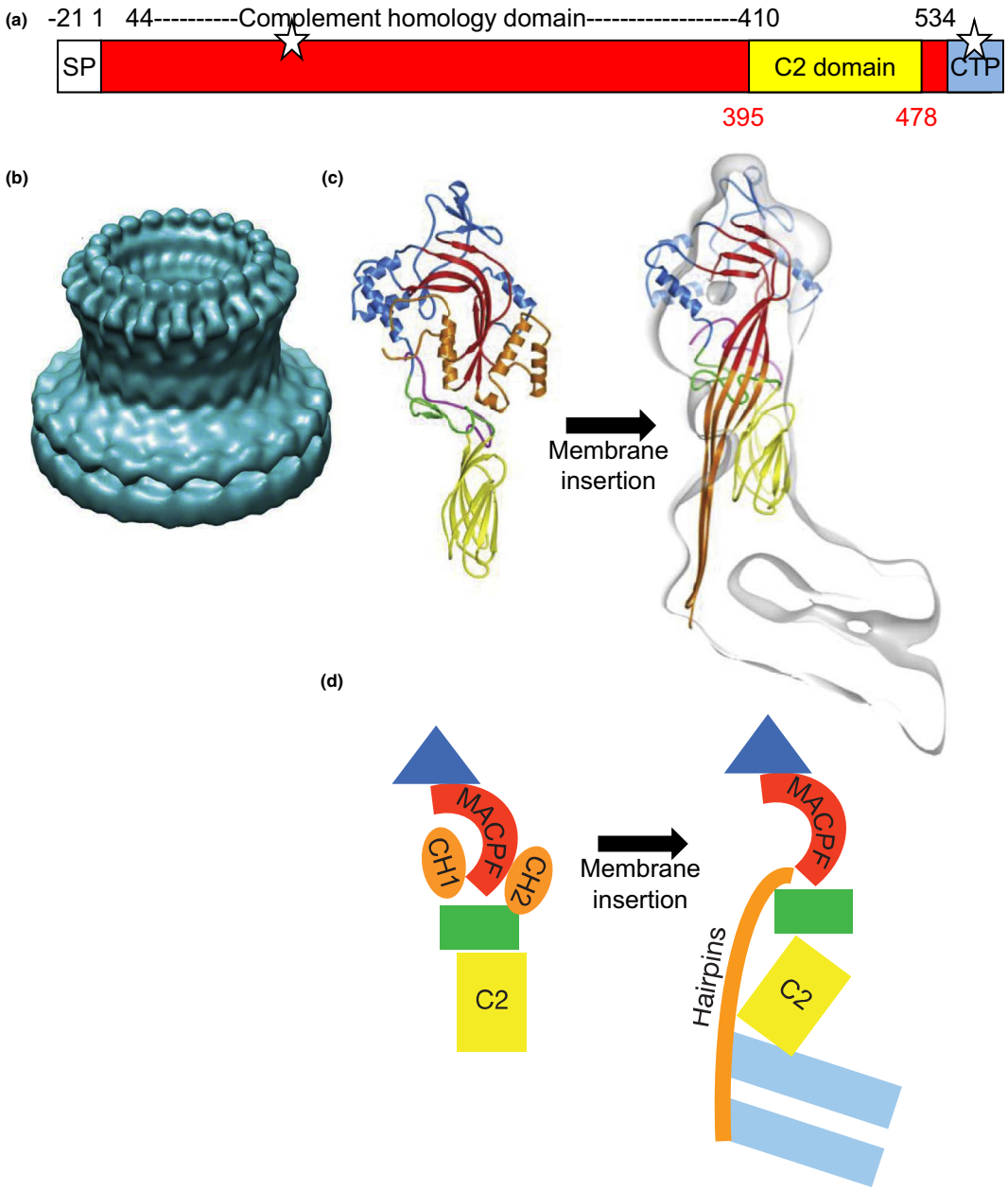

Figure 6 Perforin. (a) Perforin belongs to a family of pore-forming proteins that is homologous to complement proteins. It has a calcium-binding C2 domain that initiates pore formation by inserting in the target membrane. Stars indicate glycosylation sites. (b) The perforin subunits then assemble in the membrane to form a pore, whose structure shown here was modeled by cryoelectron tomography. (c,d) When the perforin monomers insert in the membrane and multimerize they undergo a radical conformational change. (c) shows the crystal structure of the perforin monomer (without its C-terminal domain) on the left and its structure within the pore, inferred from the structure of the pore shown in (b). The diagram in (d) shows the rearrangement of the domains of the protein during this process (MACPF, the shared domain of the complement membrane attack complex (MAC) and perforin, which forms the core of this membrane 'hole punch'; CH1 and CH2, α-helices that unwind to form hairpins during pore formation; the C2 domain). This figure is based on Law, R.H., et al., 2010. The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature 468, 447–451 and is reprinted with permission.

coagulation and degrade extracellular matrix to facilitate the movement of killer cells in tissue (Froelich et al., 2009). Low amounts of granzymes are detected in the serum of healthy donors. During inflammation and infection, elevated granzyme levels are found in serum and other bodily fluids. Although extracellular granzymes are not likely to get into the cytoplasm of cells to induce cell death without a high local concentration of perforin, they can proteolyze cell surface receptors or extracellular proteins. Recent studies suggest extracellular granzymes A and K activate macrophages to produce and secrete inflammatory cytokines, although the mechanism for this is not known and the physiological significance of these extracellular activities

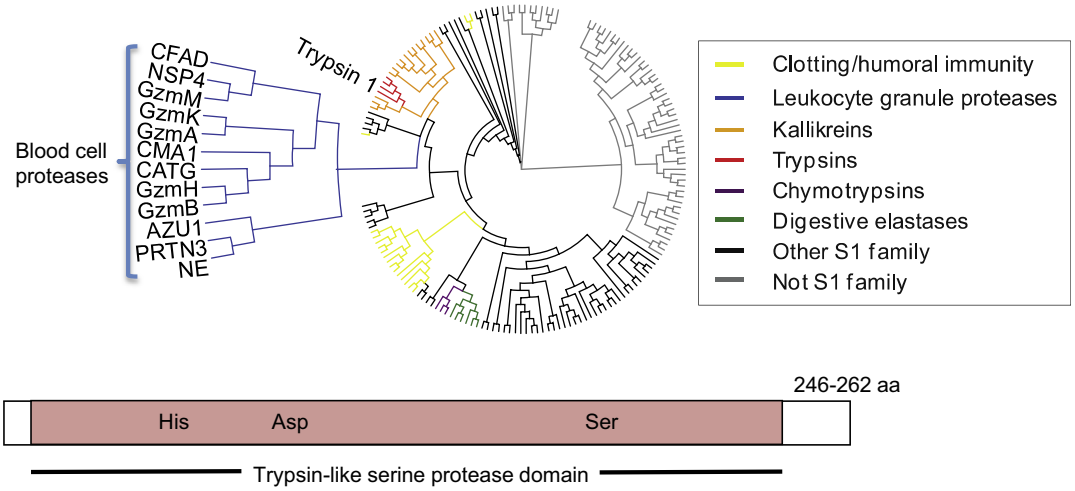

Figure 7 Granzymes. The granzymes are trypsin-like serine proteases that form a family with other proteases in blood cells. The homology between the human trypsin-like proteases is shown. They all contain a conserved catalytic triad (His–Asp–Ser), but the sequence near their active site differs. These differences are responsible for their enzymatic specificities – granzymes A and K cleave after the basic amino acids Arg or Lys, granzyme B cleaves after Asp, granzyme M after Met and granzyme H after hydrophobic Try and Phe amino acids. Adapted from Thomas, M.P., et al., 2014. Leukocyte protease binding to nucleic acids promotes nuclear localization and cleavage of nucleic acid binding proteins. J. Immunol. 192, 5390–5397 and reprinted with permission.

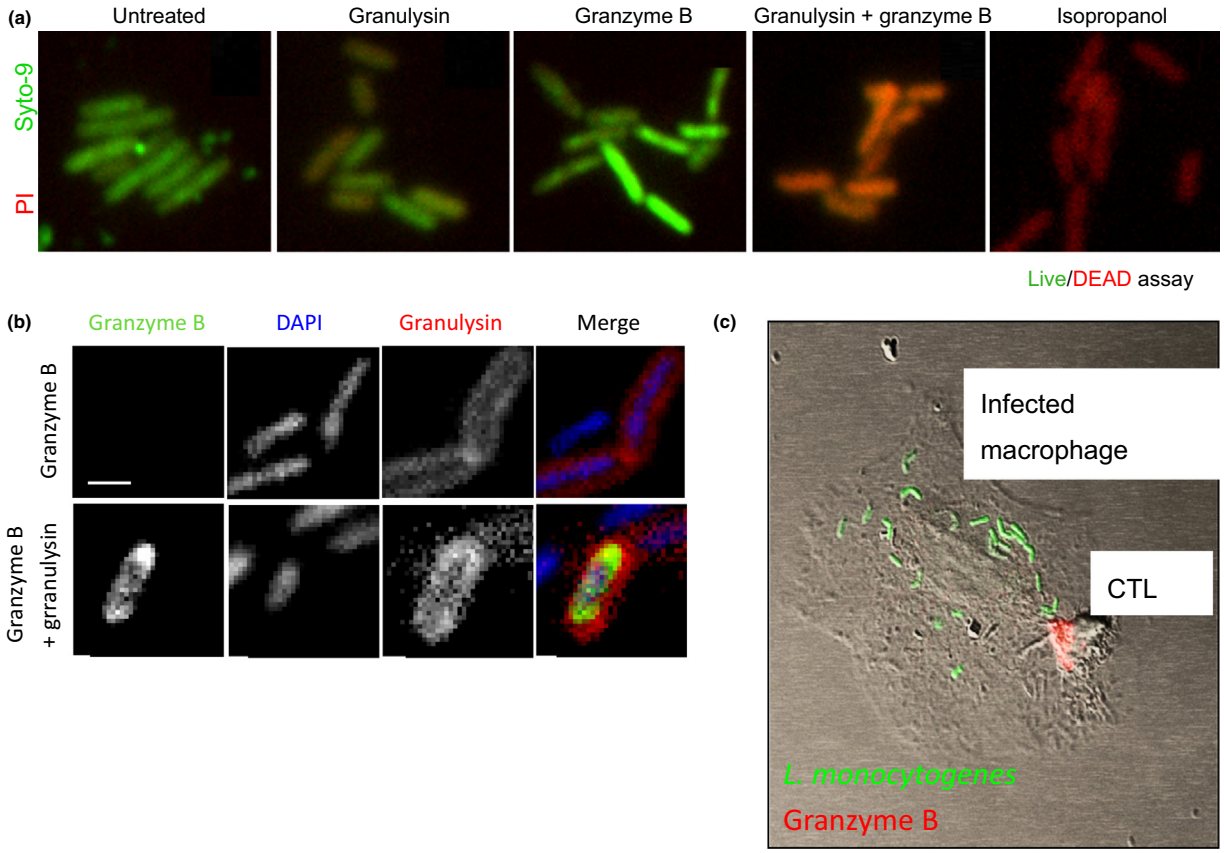

Figure 8 Granulysin delivers granzymes into bacteria. (a) When *Escherichia coli* are incubated with granulysin and granzyme B, they are killed. Bacteria were stained with two dyes – a green dye Syto9 that enters both live and dead bacteria and a red dye (propidium iodide that only enters dead cells. (b) Granzyme B does not get into bacteria on its own, but is delivered into *E. coli* by granulysin. Granulysin stays on the bacterial membranes. (c) A micrograph showing a *Listeria*-specific $CD8^+$ CTL attacking a human macrophage that is infected with green-stained *Listeria monocytogenes* bacteria. The CTL granules, stained for granzyme B, are lined up at the immune synapse. Panels (a, b) are from Walch, M., et al., 2014. Cytotoxic cells kill intracellular bacteria through granulysin-mediated delivery of granzymes. Cell 157, 1309–1323.

is still unclear. One provocative study found a dramatic granzyme B–dependent, but perforin-independent, increase in atherosclerosis complications in mice, suggesting that extracellular granzyme B might exacerbate atherosclerosis (Chamberlain et al., 2010).

Granulysin

Human cytotoxic granules contain another effector molecule – the membrane-perturbing saposin-like molecule granulysin (Krensky and Clayberger, 2009). The granulysin gene (*GNLY*) was first identified as a late activation gene expressed 3–5 days after T cell activation of naïve T cells, at the same time as the other cytotoxic effector molecule genes. Granulysin is expressed by killer cells of many mammals, but not in rodents. Its expression is limited to killer cells. Granulysin is synthesized as a precursor protein that is cleaved at both ends to produce an active peptide. The larger form is secreted by NK cells and CTLs, while the shorter form is stored and released from cytotoxic granules during NK cell or CTL attack. While perforin is only active in cholesterol-containing membranes, granulysin preferentially disrupts membranes that lack cholesterol, namely the membranes of microbes - bacteria, fungi and parasites. Granulysin plays a key role in killer cell defense against nonviral infections. (Krensky and Clayberger, 2009; Stenger et al., 1998) Perforin delivers granzymes and granulysin into target cells infected with bacteria or parasites and then granulysin delivers the granzymes into these intracellular microbes to cause their death (Walch et al., 2014; Dotiwala et al., 2016; **Figures 8** and **9**). Microbial death is largely oxidative – superoxide anion is generated as in mammalian cells, and microbial antioxidant defense enzymes are inactivated to interfere with the microbe's ability to resist killing. Microbial death occurs very rapidly – within minutes – before the host cell is killed, which limits the spread of infection to neighboring cells. Mice engineered to express granulysin in their killer cells are able to survive microbial infections that are lethal to wild-type mice, emphasizing the importance of this recently described microbial immune defense.

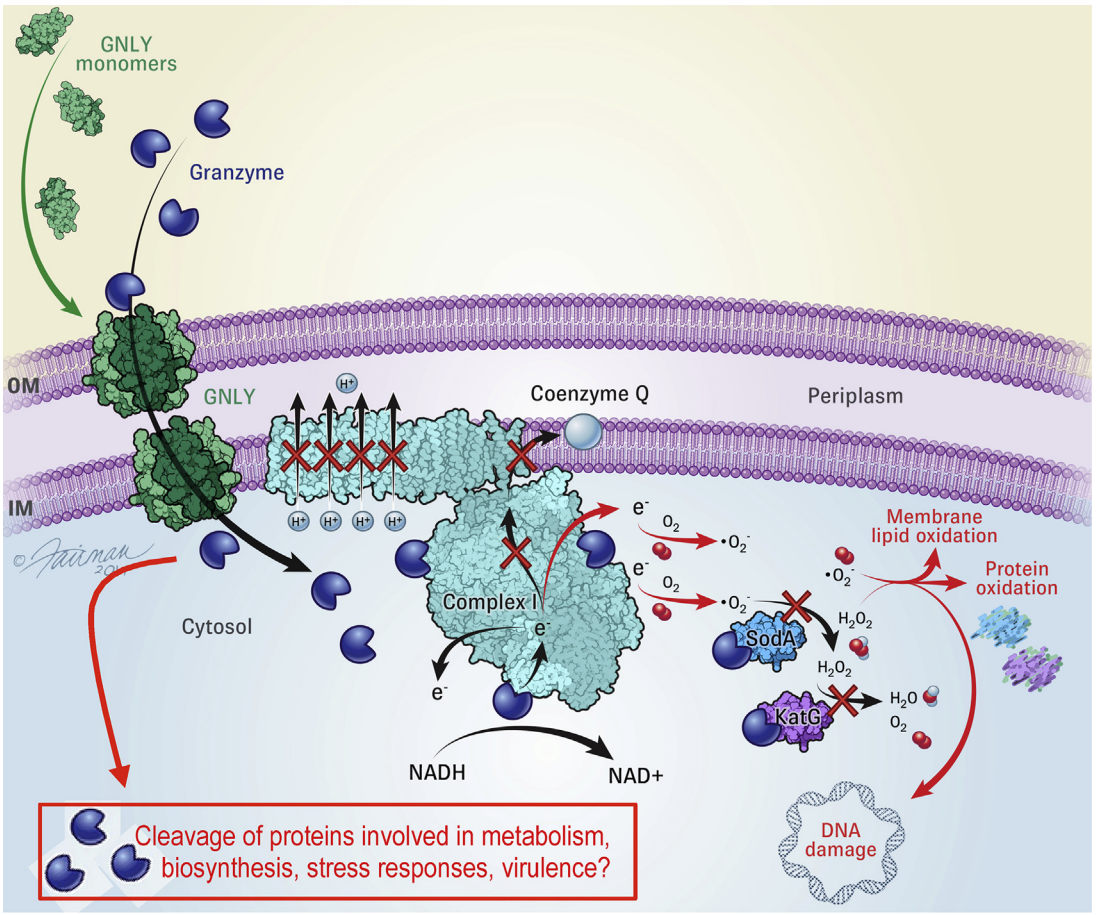

Figure 9 Model of bacterial death by granzymes and granulysin. When killer cells attack bacteria, granulysin (GNLY) forms pores in the bacterial outer and inner membranes (OM, IM) that deliver the granzymes into the bacteria. The granzymes attack electron transport chain complex I which diverts the flow of electrons to cause the formation of superoxide anion ($\bullet O_2^-$). Superoxide anion is normally detoxified by superoxide dismutases (Sod) and catalases (Kat). The granzymes interfere with bacterial oxidative stress defense against superoxide anion by cleaving and inactivating these enzymes. Thus, bacterial death is primarily oxidative, although other bacterial survival pathways are also disrupted. Figure is from Walch, M., et al., 2014. Cytotoxic cells kill intracellular bacteria through granulysin-mediated delivery of granzymes. Cell 157, 1309–1323.

DR Pathways

NK cells and CTLs also trigger target cell programmed cell death by ligating and activating DRs on target cells (Wilson et al., 2009; Suda et al., 1993; Strasser et al., 2009). The DRs belong to the tumor necrosis factor receptor (TNFR) family. All the DRs contain a cytoplasmic ~80 amino acid long death domain. Death by DR ligation can be distinguished from granule-mediated cell death because it is calcium-independent, while perforin pore formation depends on calcium. The DRs on target cells form trimers when they are activated. In humans six TNFR family receptors (FAS (CD95) activated by FAS ligand, TNFR1 activated by TNF, DR 3 activated by TL1, DR4 and DR5 activated by TNF-related apoptosis-inducing ligand (TRAIL), and DR6, which has an unknown ligand) are death receptors. Their death domains recruit adaptor molecules that serve as a platform for recruiting signaling complexes (**Figure 10**). Depending on the cellular context, signaling by the death receptors can either trigger caspase-mediated apoptosis, programmed necrosis, or proliferative and inflammatory responses (Lavrik and Krammer, 2012; Han et al., 2011). In general, the receptors that predominantly recruit the adaptor Fas-associated protein with death domain (FADD) (FAS, DR4, and DR5) are more likely to trigger cell death, while signaling from receptors that associate with the other adaptor TNF type 1–associated death domain (TRADD) (TNFR1, DR3, DR6) is more likely to activate cell proliferation and inflammation. FADD recruits caspases 8 and 10 to form the death-induced signaling complex (DISC) at the cell membrane. These caspases are autoactivated in the DISC to initiate classical apoptosis. When cell death is triggered in cells in which the caspase pathway is inhibited, targeted cells undergo an alternate programmed cell death pathway termed necroptosis (Christofferson and Yuan, 2010). The relative strength of signaling that leads to death versus inflammation is determined in part by cellular expression of c-FLIP, a caspase 8 inhibitor, that is recruited to the DISC. Some tumor cells, as well as some activated T cells and NK cells, overexpress c-FLIP, making them insensitive to DR-mediated death (Johnstone et al., 2008).

Humans and mice that are genetically deficient in either FAS or its ligand are able to defend against intracellular pathogens, but develop an autoimmune syndrome called autoimmune lymphoproliferative syndrome (ALPS) (Fisher et al., 1995; Wang et al., 1999; Bidere et al., 2006; Watanabe-Fukunaga et al., 1992). FAS-mediated death is required to eliminate chronically activated T cells and contributes to elimination of self-reactive T cells. Humans bearing caspase 8 mutations have defects in T cell activation and immunodeficiency rather than autoimmunity, which highlights the importance of the nonapoptotic signaling that results from DR engagement. Humans with caspase 10 mutations develop ALPS. Mouse studies suggest that DR5 (the ortholog of human DR4 and DR5) and its ligand TRAIL, which is expressed on NK cells, may play an important role in innate immune tumor surveillance (Takeda et al., 2001). TRAIL may also participate in eliminating activated CD8 CTLs. DR5-deficient mice are prone to develop tumors and metastases in several endogenous mouse tumor models.

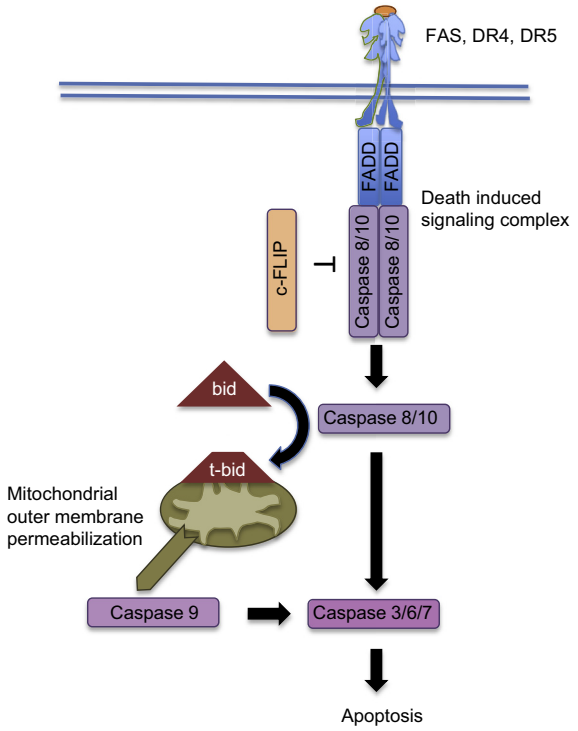

Figure 10 Death receptors. When the death receptors are activated by ligand, they form a trimer that recruits the death-induced signaling complex, which activates caspases 8 or 10. These caspases cleave bid and the truncated bid (t-bid) inserts into the mitochondrial outer membrane, permeabilizing it and causing the release into the cytosol of mitochondrial intermembrane proteins, including cytochrome C. Cytochrome C activates caspase 9, which in turn activates the effector caspases (3, 6, and 7) to cause programmed cell death. Caspases 8 and 10 can also directly activate the effector caspases. In some cells, called Type I cells, the direct activation of effector caspases by caspase 8 or 10 is potent enough to cause cell death; in other cells, called type II cells, apoptosis is only activated if death receptor signaling is amplified by the mitochondrial death pathway. A natural inhibitor of death receptor signaling is c-FLIP, which is highly expressed by some tumors. When the caspases are inhibited, death receptor signaling activates necroptosis, an inflammatory death pathway. Under some circumstances activation of these death receptors can trigger alternate signaling complexes that lead to cell proliferation and activation of NF-κB and production of inflammatory cytokines, instead of cell death.

See also: **Cells of the Innate Immune System:** Natural Killer Cells; Polymorphic KIR-HLA System Regulates Natural Killer Cell Response; The Role of Invariant NKT Cells in Immunity. **Development of T Cells and Innate Lymphoid Cells:** CD4/CD8 Lineage Commitment; DN TCRαβ Intraepithelial T Cell Development in the Thymus. **Immune Deficiency:** Apoptosis-Related Autoimmune Lymphoproliferative Syndrome; Familial Lymphohistiocytosis. **Immunity to Viral Infections:** NK Cells in Antiviral Defense; Protective and Pathogenic T Cell Responses to Virus Infections. **MHC Structure and Function:** Structure of Classical Class I MHC Molecules. **Structure and Function of Diversifying Receptors:** Structure and Function of TCRαβ Receptors. **T Cell Activation:** Recirculating and Resident Memory CD8$^+$ T Cells. **Tumor Immunology:** CD8 T Lymphocytes in Antitumor Immunity.

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