Chapter 10
Perforin: A Key Pore-Forming Protein for Immune Control of Viruses and Cancer

Jerome Thiery and Judy Lieberman

Abstract Perforin (PFN) is the key pore-forming molecule in the cytotoxic granules of immune killer cells. Expressed only in killer cells, PFN is the rate-limiting molecule for cytotoxic function, delivering the death-inducing granule serine proteases (granzymes) into target cells marked for immune elimination. In this chapter we describe our current understanding of how PFN accomplishes this task. We discuss where PFN is expressed and how its expression is regulated, the biogenesis and storage of PFN in killer cells and how they are protected from potential damage, how it is released, how it delivers Granzymes into target cells and the consequences of PFN deficiency.

Keywords Cytotoxic granules · Cytotoxicity · Cytotoxic T cells · Familial hemophagocytic lymphohistiocytosis · Granzymes · MACPF · Natural killer cells · Perforin · Pore-forming protein

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cells
ASM	Acid sphingomyelinase
CDCs	Cholesterol-dependent cytolysins
CI-MPR	Cation-independent mannose-6-phosphate receptor
c-SMAC	The central region of the immune synapse
CTL	Cytotoxic T lymphocytes
EOMES	Eomesodermin
ER	Endoplasmic reticulum

J. Thiery
INSERM U753, University Paris Sud and Gustave Roussy Cancer Campus, Villejuif, France
e-mail: jerome.thiery@gustaveroussy.fr

J. Lieberman (✉)
Program in Cellular and Molecular Medicine, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA
e-mail: judy.lieberman@childrens.harvard.edu

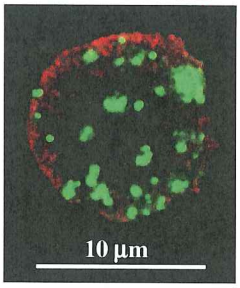
G. Anderluh and R. Gilbert (eds.), *MACPF/CDC Proteins - Agents of Defence, Attack and Invasion*, Subcellular Biochemistry 80, DOI: 10.1007/978-94-017-8881-6_10,

FHL	Familial hemophagocytic lymphohistiocytosis
GNLY	Granulysin
Gzm	Granzymes
GvHD	Graft-versus-host disease
HGH	Hemophagocytic lymphohistiocytosis
IL2	Interleukin-2
IS	Immune synapse
LCMV	Lymphochoriomeningitis virus
LCR	Locus control region
MAC	Membrane attack complex
MACPF	Membrane attack complex/perforin
MTOC	Microtubule organizing center
NK	Natural killer
PFN	Perforin
SLO	Streptolysin O
SNARE	Soluble N-ethylmaleimide-sensitive factor accessory protein receptor
TCR	T cell receptor

Introduction

Immune cytotoxic (killer) cells protect us from intracellular infection by triggering programmed cell death to eliminate infected cells; they also help protect us, although less effectively, from transformed cancer cells. The main killer cells are natural killer (NK) cells of the innate immune system and $CD8^+$ cytotoxic T lymphocytes (CTL) of adaptive immunity, although some $CD4^+$ T lymphocytes, especially T_H1 and T_{reg} cells, also deploy the specialized cell death-inducing machinery. All killer lymphocytes contain specialized secretory lysosomes, called cytotoxic granules, filled with death-inducing serine proteases, called granzymes (Gzm, "granule enzyme") (Fig. 10.1). When the killer cell recognizes a cell targeted for elimination, the cytotoxic granules move to the immune synapse (IS) formed with the target cell and fuse their membranes with the killer cell plasma membrane, dumping their contents into the IS. This process is called granule exocytosis (Figs. 10.2, 10.3). The cytotoxic granules also contain perforin (PFN), a pore-forming protein encoded by the *PRF1* gene, which has an N-terminal membrane attack complex/perforin (MACPF) domain (reviewed in [101]) that is similar to the pore forming domain of the cholesterol-dependent cytolysins (CDCs) from $Gram^+$ bacteria, such as perfringolysin O [77], and to complement components C6–C9 of the membrane attack complex (MAC) [49, 86, 98] (Fig. 10.4). PFN, which is expressed only in killer lymphocytes, delivers the death-inducing Gzms into the target cell cytoplasm. The Gzm proteases activate cell death by cleaving specific target proteins in the cytoplasm and concentrate within mitochondria and

(a)



(b)

CTL and NK cell cytotoxic granule contents

Dense core	Function
Perforin	Pore formation; internalization of granzymes
Granzymes	Serine proteases
Granulysin	Microbicidal activity
Calreticulin	Calcium storage and perforin inhibitor
Cathepsin C	Pro-granzyme processing
Serglycin	Proteoglycan matrix
periphery (resident lysosomal proteins)	
Lamp-1	
Lamp-2	Lysosomal membrane proteins
CD63	
H+ ATPase	Lysosomal acidification
Rab27a	
Munc 13-4	Granule exocytosis
VAMP7/VAMP8	
Cathepsin B	Protect CTL against perforin

Fig. 10.1 Key components of cytotoxic granules. **a** Perforin (*green*) is localized within the cytotoxic granules of a Natural Killer (NK) cell. The plasma membrane is stained *red*. **b** The cytolytic effector molecules, perforin, granzymes, and granulysin, are bound in the granule core to the serglycin proteoglycan. Calreticulin in the granule scavenges Ca^{2+} to prevent perforin membrane insertion. Cytotoxic granules also contain molecules found in all lysosomes, such as Lamp1 (CD107a), CD63 and cathepsins, as well as membrane-associated proteins specific to secretory lysosomes, such as vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory complex component (VAMP)7 or VAMP8, Munc13-4, and Rab27a, which are essential for granule exocytosis. Cathepsins B and C play a special role in cytotoxic granules—cathepsin C removes 2 N-terminal amino acids from the pro-granzymes to produce the active enzyme; lysosomal membrane-associated cathepsin B may help protect the killer cell from membrane damage in the immune synapse by cleaving and inactivating perforin. Other cathepsins may substitute for these cathepsins when they are absent or mutated

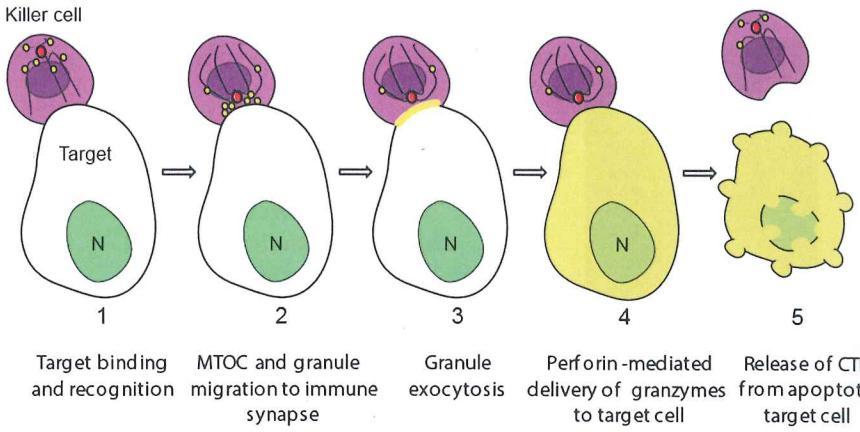


Fig. 10.2 Steps in granule-mediated cytotoxicity. After the killer cell recognizes a target cell (*1*), an immune synapse is formed at the interface and the microtubule organizing center moves to the synapse, reorganizing the microtubule network (*2*). Cytotoxic granules move along microtubules to dock at the killer cell membrane. Granule membranes fuse with the killer cell plasma membrane, releasing their contents (*yellow*) into the immune synapse (*3*). Perforin delivers the granzymes into the cytosol of target cells (*4*) where they initiate apoptotic death (*5*). The granzymes concentrate in the nucleus of target cells. The killer cell then detaches from the dying cell and is free to seek out additional targets

nuclei, where many key Gzm protein substrates reside. In the encounter with a target cell, the killer cell remains unharmed; thus killer cells are *serial* killers that can detach from one target to seek and destroy others. Target cells destroyed by cytotoxic granules die a highly regulated programmed cell death (apoptosis), rather than necrosis. Programmed cell death minimizes inflammation and damage to nearby tissue since target cells and their debris are rapidly removed by immune phagocytes, especially macrophages. Although killer lymphocytes can also activate programmed cell death by recognizing and activating death receptors on target cells, granule-mediated cell death is responsible for immune control of viral and intracellular bacterial infection and cancer.

PFN is the only molecule known to deliver the Gzms into target cells in vivo. Mice genetically deficient in *Prf1* are profoundly immunodeficient, being unable to protect themselves from viruses and prone to develop lymphoma. Humans genetically deficient in functional PFN are also impaired in their ability to handle intracellular infection and can develop an often-fatal inflammatory syndrome due to unresolved infection that can only be treated by bone marrow transplantation (see Chap. 11). Individuals bearing hypomorphic mutations that do not completely eliminate PFN function can be asymptomatic until adulthood and are susceptible to developing lymphoma. Recently a weakly paralogous protein PFN-2 that contains a MACPF domain and is expressed from the *MPEG1* gene mainly in macrophages has also been identified and is hypothesized to also form membrane pores [54]. PFN-2 may play a role in macrophage defense against bacteria.

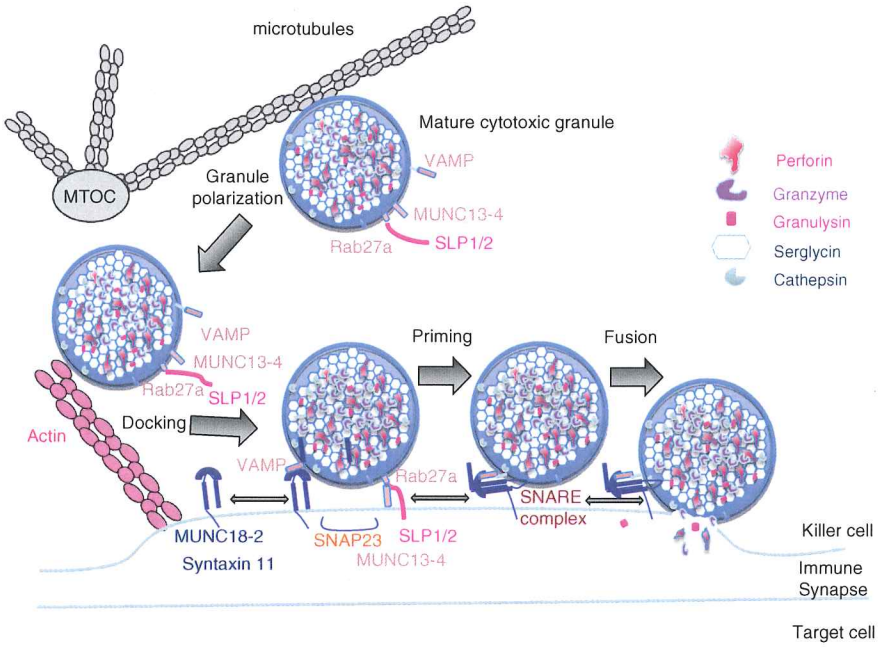
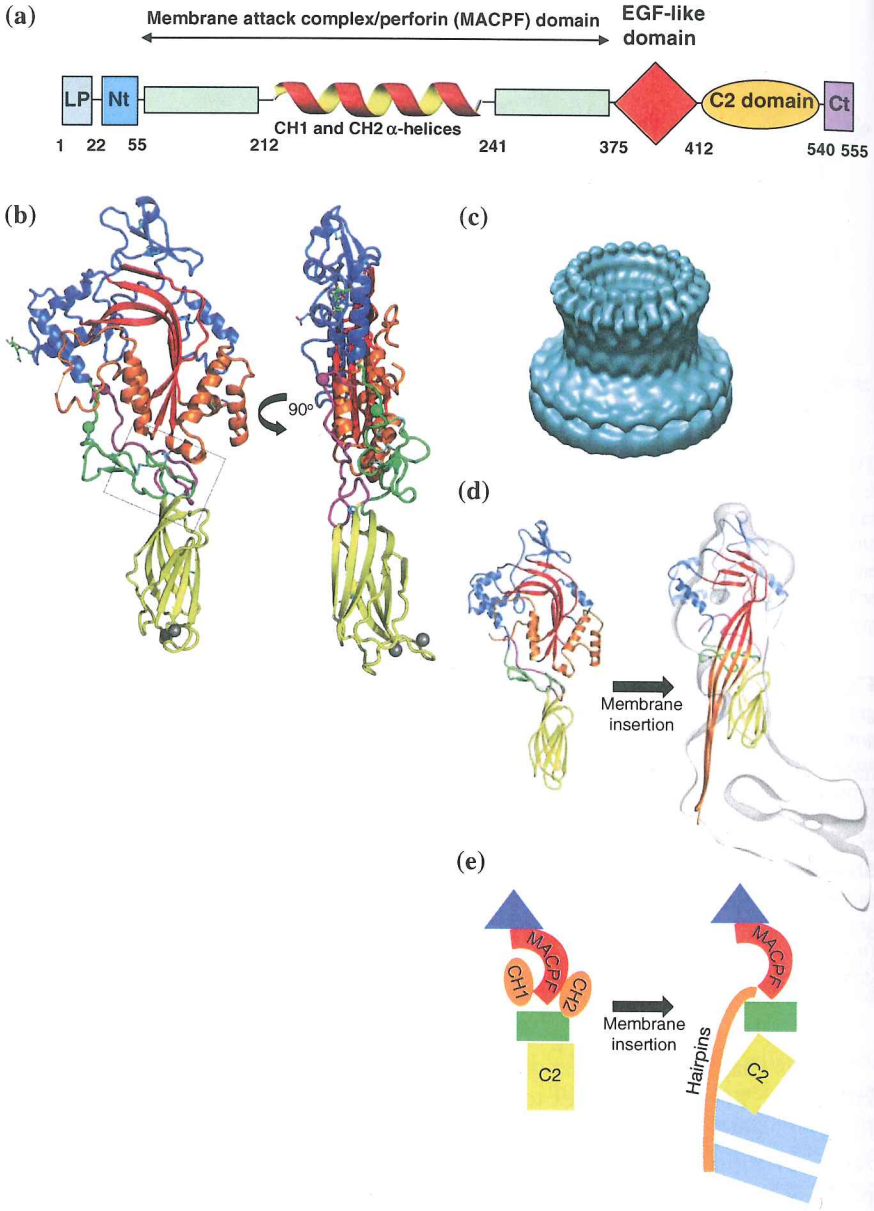


Fig. 10.3 Model of granule exocytosis. In response to antigen recognition, the mature cytotoxic granule moves along microtubules, to dock at the cell membrane at the immune synapse probably with assistance from the actin-myosin cytoskeleton. A cytotoxic granule vesicle-associated soluble N-ethylmaleimide-sensitive factor accessory (SNARE) complex component (VAMP) protein binds to Munc18-2, which is associated with plasma membrane syntaxin 11. Cytotoxic granule proteins Rab27a and Munc13-4, in association with a synaptotagmin SLP1 or SLP2, help anchor the granule to the membrane. A SNARE complex forms between plasma membrane SNAP23 and syntaxin 11 and granule membrane VAMP to initiate fusion of the granule membrane to the plasma membrane. Following membrane fusion, the cytotoxic granule contents are released into the immune synapse. After fusion, granule membrane-associated cathepsin B (*not shown*) is displayed on the killer cell membrane and protects it from perforin membrane damage. Figure adapted from [20]

In this chapter we describe our current understanding of how PFN functions in killer cells. PFN multimerizes to form pores in cholesterol-containing membranes in a Ca^{2+}-dependent manner. The structure of PFN, based on the recent crystallization of monomeric PFN and single-particle cryo-EM reconstruction of PFN pores, is described in Chaps. 4 and 6. Here we discuss where PFN is expressed and how its expression is regulated, the biogenesis and storage of PFN in killer cells and how they are protected from it, how it is released, how it delivers Gzms into target cells and the consequences of PFN deficiency. Although the simplest model for PFN-mediated Gzm delivery into target cells would be through PFN pores in the target cell plasma membrane, this model does not fit the data. Plasma membrane delivery leads to cell membrane damage and rapid target cell death by necrosis, rather than the characteristic slower and non-inflammatory immune-mediated death

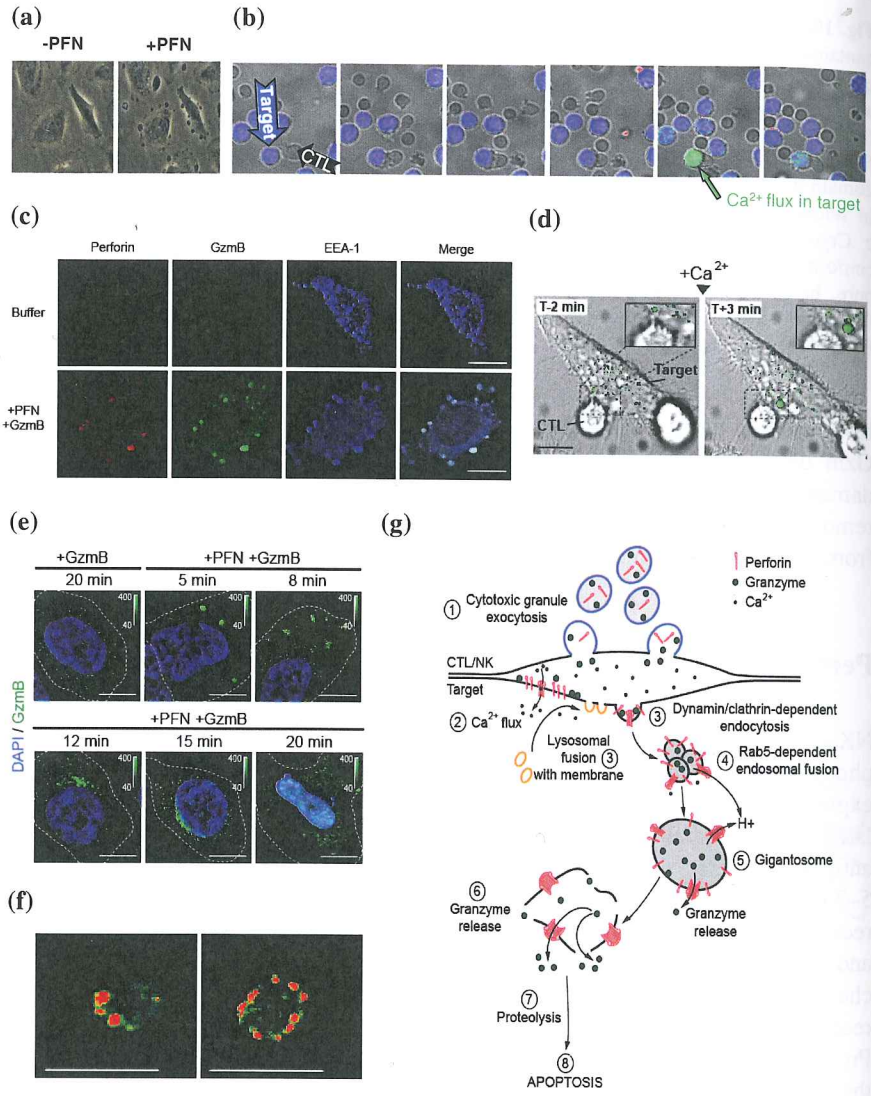

(a)
Membrane attack complex/perforin (MACPF) domain
EGF-like domain
LP
Nt
CH1 and CH2 α-helices
C2 domain
Ct
1 22 55 212 241 375 412 540 555
(b)
90°
(c)
(d)
Membrane insertion
(e)
MACPF
CH1
CH2
C2
Membrane insertion
Hairpins
MACPF
C2

◀ **Fig. 10.4** Perforin structure and insertion in the plasma membrane. **a** The PFN monomer contains an N-terminal domain (Nt) with a leader peptide (LP), followed by a membrane attack complex/perforin (MACPF) domain (composed of complement homology domains joined by two alpha-helical domains). A calcium-binding C2 domain, responsible for membrane binding, followed by a C-terminal peptide (Ct) are linked to the MACPF domain by an epidermal growth factor (EGF) domain. **b** Crystal structure of the PFN monomer, color coded with the MACPF domain in *red*, the CH1 and CH2 helices in *orange*, the EGF domain in *green*, and the C2 domain in *yellow*. The *grey balls* indicate calcium binding to the C2 domain in the crystal structure. **c** Cryoelectron microscopy reconstruction of the large PFN pore. **d**, **e** Model of PFN conformational change induced by membrane binding of the C2 domain to form a multimerized pore, based on the structure of the monomer and the reconstruction of PFN pore densities. Domains in **e** are color coded as in **b**. *Light Blue* in **e** indicates the membrane. **b–e** are adapted from [48]

by apoptosis. Here we discuss the experimental basis for an alternate model for Gzm delivery (Fig. 10.5). In this model, PFN causes transient cell membrane damage that mobilizes the target cell to repair the damaged membrane rapidly and remove PFN and Gzms on the membrane by endocytosis. Gzms are then released from endosomes when PFN forms large pores in the endosomal membrane.

Perforin is Only Expressed by Killer Lymphocytes

NK cells and $CD8^+$ T cells are the major classes of PFN-expressing killer lymphocytes. Non-lymphoid cells, B lymphocytes and noncytolytic T cells do not express PFN. Cytotoxicity and *PFN1* expression are tightly regulated and correlated. When T cells are released from the thymus and before they encounter antigen, they are "naïve"—they do not express *Prf1* and are not cytotoxic. About 5–7 days after naïve CD8 T cells encounter antigen recognized by their T cell receptor (TCR), they differentiate into effector cytotoxic cells that express PFN and some of the Gzms [36]. At the same time, they down-regulate adhesive and chemokine receptor molecules that retain them in lymph nodes and acquire receptors that allow them to traffic to tissue sites of infection and tumor invasion. Professional antigen presenting cells (APC), macrophages and dendritic cells, are the most effective cells for activating cytotoxic function. Induction of cytotoxic genes requires not only antigen-receptor activation, but also costimulation, and is greatly enhanced by APCs that have been stimulated by danger- and pathogen-associated pattern recognition receptors or by exogenous inflammatory and antiviral cytokines, including the Type I interferons, IL-1 and IFN-γ. The combination of these signals leads to production of IL-2 and other related γ_c-dependent cytokines (IL-4, IL-7, IL-9, IL-15 and IL-21), which are needed to activate PFN gene expression. This insures that cytotoxicity is only triggered by *bona fide* antigens on infected cells or cancers and not by "self" antigens that the TCR might recognize. Activated killer $CD8^+$ T cells also begin to express the Fcγ receptor CD16, also present on cytotoxic NK cells, which enables them to recognize and lyse target


(a)
-PFN
+PFN
(b)
Target
CTL
Ca2+ flux in target
(c)
Perforin
GzmB
EEA-1
Merge
Buffer
+PFN
+GzmB
(d)
+Ca2+
T-2 min
T+3 min
Target
CTL
(e)
+GzmB
+PFN +GzmB
20 min
5 min
8 min
+PFN +GzmB
12 min
15 min
20 min
DAPI / GzmB
(f)
(g)
Perforin
Granzyme
Ca2+
1 Cytotoxic granule exocytosis
CTL/NK
Target
2 Ca2+ flux
3 Dynamin/clathrin-dependent endocytosis
Lysosomal fusion 3 with membrane
4 Rab5-dependent endosomal fusion
H+
5 Gigantosome
6 Granzyme release
Granzyme release
7 Proteolysis
8 APOPTOSIS

◀ **Fig. 10.5** Model of perforin delivery of granzymes into the target cell. **a** PFN treatment of HeLa cells causes dramatic membrane perturbation and blebbing. **b** Killer cell degranulation causes a transient calcium influx in target cells that persists for a few minutes. In this experiment from [45] PHA-activated human cytotoxic T lymphocytes (CTL) were incubated with Fura-2-loaded, anti-cluster of differentiation 3 (CD3)-coated U937 cells and images were obtained every 30 s. The Fura-2 indicator dye is *blue* when calcium is low and *green* when it is elevated. **c** PFN and granzyme B are endocytosed into giant EEA-1—staining endosomes (gigantosomes). When HeLa cells are treated with PFN and granzyme B, within 5 min, granzyme B (*green*) concentrates in gigantosomes and is released beginning after about 10 min. Later the released granzyme concentrates in the target cell nucleus (*blue*). **c** is reprinted from [94]. **d** Large EEA-1^+ endosomes (*green*) form in a target cell after CTL degranulation. EGFP-EEA-1 transfected HeLa target cells were incubated with specific CTL in the absence of calcium to allow cell conjugation. After 2 min, $CaCl_2$ was added to induce CTL degranulation. Enlarged endosomes form in the target cell within minutes following CTL degranulation. **e** The addition of PFN to granzyme B causes fluorescent granzyme B (*green*) uptake into gigantosomes. After ~10 min, granzymes are released into cytosol and concentrate after 20 min in the target cell nucleus, stained with DAPI (*blue*). **f** High magnification confocal section of representative gigantosomes stained 7 min after HeLa cell treatment with human PFN showing highly localized PFN staining in clumps on the endosomal membrane. **g** Model for PFN delivery of granzymes. After cytotoxic granule exocytosis into the immunological synapse (*1*), PFN multimerizes in the target-cell membrane to form small pores through which Ca^{2+} enters (*2*), triggering a plasma membrane repair response (*3*) in which lysosomes fuse with the damaged plasma membrane and PFN and granzymes are rapidly internalized by dynamin and clathrin-dependent endocytosis. PFN and granzyme-containing endosomes then fuse together by rapid Rab5-dependent homotypic fusion in response to the transient Ca^{2+} flux (*4*) to form gigantosomes. Within gigantosomes, PFN continues to multimerize to form new and possibly bigger pores, preventing acidification and causing some granzyme release (*5*), before inducing endosomal rupture and complete granzyme release into the target-cell cytoplasm (*6*), where they initiate proteolysis (*7*) leading to programmed cell death (*8*). **d–g** are reprinted from [93]. Scale bars 10 μm (**c–e**) or 5 μm (**f**)

cells that have been coated with antibodies in a process called antibody-dependent cell-mediated cytotoxicity (ADCC). In situations of persistent and extensive antigenic stimulation, however, such as occurs in tumors and chronic viral infection, many effector T cells with surface protein expression of $CD8^+$ no longer express PFN and are not cytotoxic [97, 108, 112]. Effector $CD8^+$ T cells that lack cytotoxicity are termed "exhausted".

PFN is also present in NKT cells (a group of T cells co-expressing a TCR and NK cell surface receptors [63]). Some murine $CD4^+$ T cells, especially T_H1 cells generated by viral infection, also express *Prf1* mRNA, but about 20 times less than activated $CD8^+$ T cells [12, 13]. Effector-like $\gamma\delta$ T cells and human $CD4^+CD25^+$ regulatory T cells [33] also express PFN and can be cytotoxic.

Most effector cells in an immediate immune response die within a few weeks, but some survive and develop into memory cells. Memory cells down-regulate cytotoxic effector proteins, but the kinetics of down-regulation varies with the molecule and with the particularities of the immunostimulatory environment [19, 78]. PFN, as the limiting molecule for cytotoxicity, is down-regulated more rapidly than other effector molecules. Memory $CD8^+$ T cells rapidly reacquire cytotoxic capability within hours of restimulation. The molecular basis for this

rapid response is not well understood, although recent studies suggest that in memory $CD8^+$ T cells the chromatin of cytolytic effector gene promoters and of eomesodermin, the master transcription factor that regulates $CD8^+$ T cell effector genes, bear epigenetic marks that poise them for transcription compared to naïve T cells [1, 2, 24, 62, 113]. These cells might also store *Prf1* and *Gzm* mRNAs that can be rapidly translated upon activation.

It takes a week to ten days for naïve $CD8^+$ T cells to proliferate and differentiate into a large population of antigen-specific CTLs. In the meantime, NK cells mediate the immediate response to intracellular infection in individuals that have not been vaccinated or previously exposed. NK activating receptors recognize cell surface changes on tumors, stressed cells and infected cells, such as down-regulation of MHC/HLA molecules or cell surface expression of nonclassical MHC molecules, such as MICA and MICB, that are induced by stress. Freshly minted NK cells (at least in mice, but not studied in humans) do not immediately express Gzms and PFN [26]. They constitutively express mRNAs for *Gzma* and *Gzmb* and *Prf1*, but only have detectable GzmA protein. Hence, they have limited cytotoxicity. However, PFN and Gzms and cytotoxicity are up-regulated rapidly when NK cell activating receptors are engaged. Less differentiated NK cells that highly express the neural cell adhesion molecule NCAM or CD56 and lack CD16 are weakly or not cytotoxic, while more differentiated $CD56^{dim}CD16^+$ NK cells are potent killer cells [100]. Once an NK cell has acquired cytolytic activity, the *PRF1* gene is thought to be constitutively transcribed [79]. In the circulation $CD56^{dim}$ NK cells have about a log more PFN than $CD56^{bright}$ NK cells.

Perforin Gene Regulation

In mammals and marsupials, PFN is encoded by a single-copy gene, but multiple variants have been described in fish and amphibians, most likely as a result of genome duplication [9]. *PRF1* is closely related in sequence to the terminal complement genes in fish and other species. In mammals another more weakly related pore-forming protein, found in macrophages (MPEG-1, 12 % amino acid identity), may have been a *PRF1* ancestor, since it can be traced evolutionarily to sponges [54]. Sequence alignments show a high degree of conservation of *Prf1* genes from different species. This high conservation may be related to its non-redundant function as a target cell membrane damaging agent, essential for cytotoxicity. Despite a 30 % divergence in sequence, mouse and human PFN can functionally substitute for each other.

Both human *PRF1* and mouse *Prf1* genes are located on chromosome 10 and share a common simple structure comprised of three small exons that span 6 kb [70] as well as 5′- and 3′-untranslated regions. The core promoter and sites of transcription initiation have been mapped, as well as cis-acting functional sequences in the proximal region of the *PRF1* gene. The *PRF1* promoter is GC-rich, does not possess a TATA-box and has one major and several minor sites

of transcription initiation [50]. The promoter is moderately active and not specific for killer cells. However, basal transcriptional activity is repressed in non-cytotoxic cells by a sequence upstream by −240 bp and an unidentified transcriptional repressor [115]. The two key transcription factors, T-bet (*TBX21*) and eomesodermin (*EOMES*), that belong to the T-box family are the key master regulators of cytotoxic gene expression and survival of committed $CD8^+$ memory cells [31, 67, 90, 107]. After naïve $CD8^+$ T cell activation, T-bet is induced before eomesodermin [18]. Notch signaling and the Runx3 transcription factor upregulate *Eomes*, but also directly upregulate expression of *Prf1* [17, 18]. Mice deficient in both *Tbx21* and *Eomes* genes are unable to control tumors and intracellular infection [5, 40, 117]. They develop a wasting syndrome caused by anomalous differentiation to IL-17-secreting cells, suggesting that these two transcription factors not only positively regulate cytotoxic gene expression and other genes required for CTL survival and function, but also suppress differentiation to alternate lineages. An uncharacterized Ets family transcription factor also supports *PRF1* promoter activity in killer cells [110].

Two enhancers at −15 and −1 kb have also been described. Interleukin-2 (IL2) signalling induces the STAT-5 transcription factor, which stimulates these enhancers, leading to expression of both human *PRF1* and mouse *Prf1* genes [114]. The −1 kb enhancer links *PRF1* gene expression to signalling from other cytokines through STAT3 or STAT4 [109, 111]. Additional transcription factors can also bind to the −15 and −1 kb enhancers including NF-κB, NFAT, Ikaros and AP-1 [116]. However, the presence of these two enhancers is not sufficient for optimal and physiological expression of PFN. PFN expression is controlled by an extended 150 kb cis-regulatory "territory" that includes a locus control region (LCR) regulating the developmental and activation-specific expression of PFN only in T cells and NK cells [69].

Perforin Structure, Mechanism of Membrane Insertion and Pore Formation

Mature PFN is a 533 amino acid protein consisting of three domains: an N-terminal MACPF domain, an intermediate EGF-like domain and a C-terminal Ca^{2+}-binding C2 domain responsible for the initial Ca^{2+}-dependent binding of PFN to membrane surfaces (Fig. 10.4). At high concentrations PFN multimerizes in a cholesterol- and Ca^{2+}-dependent manner in the plasma membrane of cells to form 5–15 nm pores [48, 59, 71, 81, 98]. Recent crosslinking and biophysical studies suggest that PFN may form at least two types of pores in membranes—small unstable pores composed of about seven monomers, and much larger stable pores [72, 93]. Cryoelectron microscopy reconstructions suggest that the large pores are composed of ~19–24 subunits and have a lumen large enough for Gzm monomers or GzmA dimers to pass through easily. Membrane pore formation by PFN can be separated into three stages, each involving structural transitions: membrane binding, multimerization

and formation of a transmembrane pore. PFN mainly forms pores in cholesterol-containing membranes. However, it can also less efficiently form pores in cholesterol poor membranes that are found in bacteria, fungi and parasites [72]. It is largely inactive against pathogens, which do not synthesize cholesterol.

The PFN C2 domain, which initiates docking with the lipid membrane in a Ca^{2+}-dependent manner, contains key aspartic acid residues that coordinate binding of up to four Ca^{2+} atoms [105]. The CDCs contain a Thr-Leu motif that binds to cholesterol [25] and causes two nearby hydrophobic loops to insert into the lipid bilayer and anchor the CDC monomer perpendicularly to the membrane surface [74]. However, PFN lacks this motif.

The N-terminal MACPF region of the mature 67 kDa protein (residues 44–410 of the human protein) is homologous to domains in complement proteins C6, C7, C8α, C8β and C9 that form the complement MAC. The crystal structure of monomeric mouse PFN was recently solved [48]. The MACPF domain is similar in structure to that of bacterial pore-forming cholesterol-dependent cytolysins, although they have been hypothesized to insert into membranes in opposite orientations (although this is uncertain [30]). The PFN (and CDC) MACPF domain are composed of a bent and twisted four-stranded β-sheet flanked by clusters of α-helices. During pore formation the α-helices unwind to insert into lipid membranes as amphipathic β-strands, which assemble into a transmembrane β-barrel during multimerization [75, 83]. PFN multimerization involves the association of oppositely charged residues on two "flat faces" of the MACPF domain (R213 and E343) [7]. Between the N-terminal MACPF domain and the C2 domain, a central EGF-like domain lies. Little is known about the function of this domain. However, in the PFN monomer, the EGF-like domain interacts with the α-helices of the MACPF domain and may help fix their orientation to the membrane [48].

Perforin Synthesis and Storage

PFN is translated with a leader sequence that directs it to the endoplasmic reticulum (ER), but because it could potentially cause self-destruction of killer cells, the biosynthesis and storage of PFN is carefully controlled. PFN only forms pores at neutral pH, which means that the neutral, Ca^{2+}-rich ER milieu would be ideal for activating it. The inactivity of PFN in the ER was initially ascribed to its synthesis as an inactive precursor, which is only activated by removal of a carboxy-terminal glycosylated peptide in cytotoxic granules [99]. Removal of this C-terminal peptide was proposed to be necessary for Ca^{2+} to bind to the C2 domain to enable membrane binding. However, this idea is not supported by the solved structure of monomeric PFN in which the C-terminal peptide and the C2 domain are not close enough to interact [48]. More recent data show that PFN is active within the ER, but that glycosylation at 2 sites, in the MACPF domain and in the C-terminal peptide, leads to its rapid trafficking to the Ca^{2+}-poor Golgi and thence to safe storage in acidic cytotoxic granules [11]. A conserved C-terminal

tryptophan residue, working through an unknown mechanism, facilitates the rapid transport from the ER to the Golgi [11]. Mutation of the terminal tryptophan leads to enhanced death of the killer cell. In the ER and cytotoxic granules, PFN interacts with calreticulin, an ER chaperone and Ca^{2+}-binding protein, which may also inhibit PFN membrane insertion until after the granules are released [21, 27].

Cytotoxic granules are acidic, electron-dense, specialized secretory lysosomes [68] (Fig. 10.1). These granules are mobilized like secretory vesicles in other secretory cells, such as neurosecretory vesicles near the synapses of neurons and melanin-containing vesicles of melanocytes. The acidic environment of the granules not only inhibits PFN binding to Ca^{2+} [105], but also contributes to PFN stability. Indeed, PFN levels in CTLs treated with concanamycin, an inhibitor of the vacuolar H^+-ATPase, which disrupts granule acidification, are dramatically decreased [44]. In addition to PFN, cytotoxic granules contain the Gzms and a cationic pore-forming molecule, granulysin (GNLY), which is homologous to the saposins and selectively active at disrupting negatively charged bacterial and possibly fungal and parasite cell membranes. GNLY is expressed in humans and nonhuman primates and orthologues are found in some other species (pigs, cows and horses), but not in rodents. Like PFN, GNLY expression is restricted to cytotoxic cells. The positively charged cytotoxic effector molecules are bound in the granule to an acidic proteoglycan, called serglycin, after its many Ser-Gly repeats [53, 57], which has been proposed as another mechanism for reducing PFN multimerization within the granules [29]. However, although the killer cells of serglycin-deficient mice store less GzmB, they have normal amounts of PFN and GzmA and have unimpaired cytotoxicity [34].

In addition to these specialized molecules, the cytotoxic granules also contain lysosomal enzymes, the cathepsins, and internal lysosomal membrane proteins, such as CD107 (Lamp1). The outside of the granule membrane binds SNARE (soluble N-ethylmaleimide-sensitive factor accessory protein receptor) proteins, synaptotagmins and Rab GTPases, that regulate vesicular trafficking and cytotoxic granule release. Some of these molecules, including Rab27a and Munc13-4, which are important for granule exocytosis, are only incorporated into cytotoxic granules as they mature by fusion of cytotoxic granules with specialized exocytic vesicles, formed in secretory cells by fusion of late endosomes and recycling endosomes (Fig. 10.3). Some of the granule-associated molecules associate with lysosomes in all cells, while some have a specialized function in killer cells.

During target cell killing, PFN is released from the cytotoxic granules into the IS formed between the target and killer cell (Fig. 10.2, see below). In these conditions, PFN is free to act on the target, but also on the effector cell plasma membrane. How the killer cell membrane is protected from PFN is still not completely clear. The granule membrane protein cathepsin B is incorporated in the killer cell plasma membrane when cytotoxic granules fuse with the killer cell plasma membrane during granule exocytosis. Cathepsin B can cleave PFN and potentially inactivate any PFN redirected toward the killer cell [4]. However, CTLs from mice deficient in cathepsin-B survive target cell encounters in vitro and in vivo [6]. A possible explanation for these seemingly contradictory results could

be that other membrane-bound granule cathepsins also inactivate PFN when cathepsin B is absent.

Perforin and Cytotoxic Protein Release into the Immune Synapse

When CTL and NK cells form an IS with a target cell, engagement of activating receptors, including the T cell receptor, killer cell activating receptors, and Fc receptors, activates the killer cell to destroy the target cell (Figs. 10.3, 10.4). Their activation for cytolysis is enhanced by binding of CD8 or CD4, costimulatory receptors and adhesion molecules like LFA-1, which cluster in well-defined concentric rings within the IS. Killer cell activation causes a Ca^{2+} flux that induces lytic granules to cluster around the microtubule organizing center (MTOC) and then align along the immunological synapse [10, 20, 22, 23, 46, 51, 64] (Figs. 10.2, 10.3). Granules move to the immune synapse via both the microtubule network and actin cytoskeleton. Cytotoxic granules then dock to the killer cell plasma membrane in the central region of the immune synapse (c-SMAC). Cytotoxic granule docking is orchestrated by binding of Rab27a on the cytosolic side of the mature granule membrane with synaptotagmin-like proteins, SLP1 or SLP2, which are anchored in the cell membrane. Docked granules are then primed for fusion by the interaction of Munc13-4 on their surface with syntaxin 11 on the killer cell membrane. This triggers the formation of a SNARE complex, the molecular machine for granule membrane fusion, between cytotoxic granule VAMP7 or VAMP8 with syntaxin 11 and SNAP23 on the cell membrane. Granule membrane fusion also requires participation of Munc18-2 to trigger the conformational activation of the SNARE complex. Acid sphingomyelinase may be required for the contraction of docked cytotoxic granules and expulsion of their contents [37]. Once in the presumably pH neutral IS, PFN is probably released from serglycin [53], and can bind Ca^{2+} to become activated for membrane insertion.

How Perforin Delivers Granzymes into Target Cells

PFN was originally hypothesized to form large pores in the target cell plasma membrane that allow Gzms to passively diffuse into the target cell. The electron microscopy images of large PFN pores in cell membranes on which this idea was based were all generated by applying very high concentrations of PFN to cells. At these concentrations, PFN causes necrotic cell death by irreversibly damaging the target cell membrane. However, during killer cell-induced death, the simple plasma membrane pore model does not fit the data (Fig. 10.5). In this cell

membrane pore model, Gzms enter the cytosol directly but during killer cell lysis or when cells are treated with the sublytic concentrations of PFN that deliver Gzms into cells to induce apoptosis, Gzms are not detected at first in the cytosol, but instead are initially endocytosed into clathrin-coated vesicles and transported to endosomes [45, 57, 93, 94].

Gzms can bind to the cation-independent mannose-6-phosphate receptor (CI-MPR) or heparin receptors on target cells [60], but cells lacking these receptors are killed just as efficiently [47, 96]. Because the cell membrane is negatively charged and the Gzms are very basic (pI $\sim$10), the Gzms bind to cell surfaces independently of any receptor via electrostatic interactions [8, 84]. Receptor independence insures that all cells can be targeted and viruses or cancer cells cannot evade immune surveillance by down-regulating a cellular receptor. In the absence of PFN, bound Gzms are inefficiently endocytosed, but because they do not escape from endocytic compartments, there is no cell death without PFN [14, 28, 85].

We found that PFN indeed forms plasma membrane pores in the target cell, but these pores are small and transient and only allow small dyes to begin to enter the cytosol before the damaged area is walled off. However, Ca^{2+} flows into the target cell through these pores and remains elevated for a few minutes. Because intracellular Ca^{2+} is low in cells with an intact cell membrane, the cell senses a Ca^{2+} influx as a sign that the plasma membrane has been breached. The elevated Ca^{2+} triggers a rapid cellular membrane damage response (also known as cellular "wound-healing" [76]) in which intracellular vesicles move to the plasma membrane and fuse with it to patch holes, removing and internalizing into endosomes any damaged membrane [39, 55, 92, 93]. Some of the damaged membrane may also be removed by blebbing. When the membrane repair response is inhibited then because the cell membrane remains leaky, target cells die by necrosis instead of by the slower, regulated, noninflammatory and energy-dependent apoptosis. Normally, however, membrane wound healing immediately activates endocytosis leading to the rapid internalization of the membrane-bound granzymes, granulysin and PFN. Elevated cytosolic Ca^{2+} activates endosomal fusion, and the resulting granzyme- and PFN-containing endosomes fuse to form giant endosomes $\sim$10 times larger than normal that have been termed gigantosomes. In the endosomal membrane, PFN forms larger and more stable pores through which Gzms begin to leak out into the cytosol. The leakiness of the endosome prevents its acidification, allowing PFN to remain active, and about 10–15 min after cell death has been triggered the gigantosomes become unstable and rupture, releasing any remaining cargo to the cytosol where they activate programmed cell death. Although endosomal uptake, gigantosome formation, rupture and Gzm release have been visualized in cells treated with sublytic PFN and Gzms and during T cell and NK cell-mediated killing, this model is controversial and some researchers still think that the original plasma membrane pore model is correct [52].

Bacterial and viral endosomolysins can substitute for PFN in vitro (and are widely used as laboratory reagents for intracellular delivery [14]) and potentially might play a similar role in vivo in limited circumstances. A similar repair of injured plasma membrane by rapid Ca^{2+}-dependent endocytosis has also been

demonstrated with the pore forming protein streptolysin O (SLO) [39]. The release of lysosomal acid sphingomyelinase (ASM) by SLO-wounded cells promotes endocytosis and membrane lesion removal [91]. When ASM is released from the injured cell, it converts sphingomyelin in the outer leaflet of the plasma membrane to ceramide [38]. PFN also induces the formation of large endosome-like invaginations in artificial liposomes [73].

The idea that PFN activity at the plasma membrane leads to pores that are either too small or too rapidly removed to deliver Gzms directly to the cytosol, but that PFN pores formed in endosomal membranes are functional for Gzm delivery to the cytosol is consistent with a recent study of PFN pore conductance and cryo-electron microcroscopy in planar lipid bilayers and unilamellar vesicles of different lipid compositions and sizes [72]. In this study two different membrane-bound PFN conformations were observed, which were interpreted as pre-pore and pore states of the protein. Small, highly unstable pores preceded the development of stably open and larger pores that retain a size distribution [72]. In another study, PFN monomers formed at the plasma membrane included arc-like structures, representing incomplete PFN pores [58]. These studies suggest that the rapid membrane repair response interferes with the formation of larger pores on the plasma membrane, restricting Gzm entry, but that PFN multimerizes into larger stable pores on the gigantosome membrane, where the membrane repair response doesn't operate.

Perforin Deficiency and Disease

Prf1 knockout mice are severely impaired in their defence against viruses and immune surveillance of tumors [3, 35, 42, 43, 87, 106]. Moreover, in response to lymphochoriomeningitis virus (LCMV), $Prf1^{-/-}$ mice develop a hemophagocytic lymphohistiocytosis (HLH) syndrome that resembles the syndrome associated with *PRF1* insufficiency in humans (also called familial hemophagocytic lymphohistiocytosis (FHL) [41, 88]). $Prf1^{-/-}$ mice are also less able to clear infection with the intracellular bacterium *Listeria monocytogenes* [42]. They are more susceptible to chemically or oncogenically induced tumors, including 3-methylcholanthrene-induced fibrosarcoma [82] and oncogene-driven mammary adenocarcinoma [89]. As they age, $Prf1^{-/-}$ mice also spontaneously develop highly aggressive disseminated B-cell lymphomas [87]. In a large cohort ($n > 800$) of aging $Prf1^{-/-}$ mice, additional tumors developed including thymic lymphomas, sarcomas, and lung adenocarcinoma. The severe immune consequences of *Prf1* deficiency can be contrasted by the mild phenotypes associated with Gzm deficiency, likely because the Gzms have redundant functions. Mice deficient in any one of the 10 Gzms, or even of the 5 Gzms in the Gzm B cluster, only have subtle differences compared to wild-type animals. Requirements for a single Gzm have been shown in some cases by specific immune challenges. For example, Gzm A-deficient mice are more susceptible to the poxvirus ectromelia [61] and Gzm B-deficient mice have

reduced graft-versus-host disease (GvHD) [32]. No human clinical syndromes have been identified with gene mutations of the 5 human Gzm genes.

In humans, *PRF1* bi-allelic mutations are extremely rare, but have been identified in 30–60 % of young children suffering from a rare autosomal recessive disorder called FHL (FHL2 or Type 2 FHL) [88] that is often fatal if not treated with bone marrow transplantation. Mutations that interfere with cytotoxic granule exocytosis (*UNC13D* mutations encoding for Munc13-4 (FHL3); *STX11* mutations encoding for syntaxin-11 (FHL4) and *STXBP2* mutations encoding for Munc18-2 (FHL5)) cause the same syndrome. The genetic defect in some cases has not been identified (FHL1). Killer cell cytotoxicity is profoundly impaired in these patients. FHL is an immune homeostasis disorder characterized by uncontrolled activation and proliferation of $CD4^+$ and $CD8^+$ T cells and macrophage activation [101], which is likely secondary to chronic unresolved viral infection, especially of herpesviruses. Macrophage activation is driven by excessive production of IFN-γ by activated $CD8^+$ T cells [65, 66]. In these patients, activated macrophages in the spleen and bone marrow phagocytose erythrocytes, leukocytes, and platelets, resulting in severe pancytopenia and anemia. Moreover, the uncontrolled secretion of inflammatory cytokines (IL-1, IL-6, and TNF-α) by these activated macrophages results in severe fever and other symptoms.

Sequencing of *PRF1* mutations in FHL patients has identified nonsense, frameshift and missense mutations that disrupt PFN synthesis, folding or activity [102, 104]. Some patients with hypomorphic bi-allelic mutations that affect PFN folding or stability have milder disease that is not diagnosed until adulthood. These patients are prone to develop leukemia and lymphoma [16]. The A91 V allele (the most common *PRF1* genetic variant, found in 3–17 % of Caucasians) leads primarily to misfolding of PFN rather than complete loss of function [95, 105]. The killer cells of patients with A91 V mono or bi-allelic mutations have reduced cytolytic activity [103]. A91 V mono or bi-allelic mutations predispose to acute lymphoblastic leukemia [80], anaplastic large cell lymphoma [15] and BCR-ABL^+ acute lymphoblastic leukemia [56].

References

1. Araki Y, Fann M, Wersto R, Weng NP (2008) Histone acetylation facilitates rapid and robust memory CD8 T cell response through differential expression of effector molecules (eomesodermin and its targets: perforin and granzyme B). J Immunol 180:8102–8108
2. Araki Y, Wang Z, Zang C, Wood WH 3rd, Schones D, Cui K, Roh TY, Lhotsky B, Wersto RP, Peng W, Becker KG, Zhao K, Weng NP (2009) Genome-wide analysis of histone methylation reveals chromatin state-based regulation of gene transcription and function of memory CD8+ T cells. Immunity 30:912–925
3. Badovinac VP, Hamilton SE, Harty JT (2003) Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. Immunity 18:463–474
4. Balaji KN, Schaschke N, Machleidt W, Catalfamo M, Henkart PA (2002) Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. J Exp Med 196:493–503

5. Banerjee A, Gordon SM, Intlekofer AM, Paley MA, Mooney EC, Lindsten T, Wherry EJ, Reiner SL (2010) Cutting edge: the transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. J Immunol 185:4988–4992
6. Baran K, Ciccone A, Peters C, Yagita H, Bird PI, Villadangos JA, Trapani JA (2006) Cytotoxic T lymphocytes from cathepsin B-deficient mice survive normally in vitro and in vivo after encountering and killing target cells. J Biol Chem 281:30485–30491
7. Baran K, Dunstone M, Chia J, Ciccone A, Browne KA, Clarke CJ, Lukoyanova N, Saibil H, Whisstock JC, Voskoboinik I, Trapani JA (2009) The molecular basis for perforin oligomerization and transmembrane pore assembly. Immunity 30:684–695
8. Bird CH, Sun J, Ung K, Karambalis D, Whisstock JC, Trapani JA, Bird PI (2005) Cationic sites on granzyme B contribute to cytotoxicity by promoting its uptake into target cells. Mol Cell Biol 25:7854–7867
9. Bischofberger M, Gonzalez MR, van der Goot FG (2009) Membrane injury by pore-forming proteins. Curr Opin Cell Biol 21:589–595
10. Bossi G, Booth S, Clark R, Davis EG, Liesner R, Richards K, Starcevic M, Stinchcombe J, Trambas C, Dell'Angelica EC, Griffiths GM (2005) Normal lytic granule secretion by cytotoxic T lymphocytes deficient in BLOC-1, -2 and -3 and myosins Va, VIIa and XV. Traffic 6:243–251
11. Brennan AJ, Chia J, Browne KA, Ciccone A, Ellis S, Lopez JA, Susanto O, Verschoor S, Yagita H, Whisstock JC, Trapani JA, Voskoboinik I (2011) Protection from endogenous perforin: glycans and the C terminus regulate exocytic trafficking in cytotoxic lymphocytes. Immunity 34:879–892
12. Brown DM, Dilzer AM, Meents DL, Swain SL (2006) CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J Immunol 177:2888–2898
13. Brown DM, Kamperschroer C, Dilzer AM, Roberts DM, Swain SL (2009) IL-2 and antigen dose differentially regulate perforin- and FasL-mediated cytolytic activity in antigen specific CD4 + T cells. Cell Immunol 257:69–79
14. Browne KA, Blink E, Sutton VR, Froelich CJ, Jans DA, Trapani JA (1999) Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol 19:8604–8615
15. Cannella S, Santoro A, Bruno G, Pillon M, Mussolin L, Mangili G, Rosolen A, Arico M (2007) Germline mutations of the perforin gene are a frequent occurrence in childhood anaplastic large cell lymphoma. Cancer 109:2566–2571
16. Chia J, Yeo KP, Whisstock JC, Dunstone MA, Trapani JA, Voskoboinik I (2009) Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. Proc Natl Acad Sci USA 106:9809–9814
17. Cho OH, Shin HM, Miele L, Golde TE, Fauq A, Minter LM, Osborne BA (2009) Notch regulates cytolytic effector function in CD8+ T cells. J Immunol 182:3380–3389
18. Cruz-Guilloty F, Pipkin ME, Djuretic IM, Levanon D, Lotem J, Lichtenheld MG, Groner Y, Rao A (2009) Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. J Exp Med 206:51–59
19. Cui W, Kaech SM (2010) Generation of effector CD8+ T cells and their conversion to memory T cells. Immunol Rev 236:151–166
20. de Saint Basile G, Menasche G, Fischer A (2010) Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. Nat Rev Immunol 10:568–579
21. Dupuis M, Schaerer E, Krause KH, Tschopp J (1993) The calcium-binding protein calreticulin is a major constituent of lytic granules in cytolytic T lymphocytes. J Exp Med 177:1–7
22. Dustin ML (2009) The cellular context of T cell signaling. Immunity 30:482–492
23. Dustin ML, Long EO (2010) Cytotoxic immunological synapses. Immunol Rev 235:24–34
24. Fann M, Godlove JM, Catalfamo M, Wood WH 3rd, Chrest FJ, Chun N, Granger L, Wersto R, Madara K, Becker K, Henkart PA, Weng NP (2006) Histone acetylation is associated

with differential gene expression in the rapid and robust memory CD8(+) T-cell response. Blood 108:3363–3370
25. Farrand AJ, LaChapelle S, Hotze EM, Johnson AE, Tweten RK (2010) Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface. Proc Natl Acad Sci USA 107:4341–4346
26. Fehniger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, French AR, Ley TJ (2007) Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity 26:798–811
27. Fraser SA, Karimi R, Michalak M, Hudig D (2000) Perforin lytic activity is controlled by calreticulin. J Immunol 164:4150–4155
28. Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, Hanna W (1996) New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem 271:29073–29079
29. Galvin JP, Spaeny-Dekking LH, Wang B, Seth P, Hack CE, Froelich CJ (1999) Apoptosis induced by granzyme B-glycosaminoglycan complexes: implications for granule-mediated apoptosis in vivo. J Immunol 162:5345–5350
30. Gilbert RJ, Mikelj M, Dalla Serra M, Froelich CJ, Anderluh G (2013) Effects of MACPF/CDC proteins on lipid membranes. Cell Mol Life Sci 70:2083–2098
31. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM (2004) Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol 4:900–911
32. Graubert TA, Russell JH, Ley TJ (1996) The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. Blood 87:1232–1237
33. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity 21:589–601
34. Grujic M, Braga T, Lukinius A, Eloranta ML, Knight SD, Pejler G, Abrink M (2005) Serglycin-deficient cytotoxic T lymphocytes display defective secretory granule maturation and granzyme B storage. J Biol Chem 280:33411–33418
35. Gupta M, Greer P, Mahanty S, Shieh WJ, Zaki SR, Ahmed R, Rollin PE (2005) CD8-mediated protection against Ebola virus infection is perforin dependent. J Immunol 174:4198–4202
36. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA (1997) Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 186:1407–1418
37. Herz J, Pardo J, Kashkar H, Schramm M, Kuzmenkina E, Bos E, Wiegmann K, Wallich R, Peters PJ, Herzig S, Schmelzer E, Kronke M, Simon MM, Utermohlen O (2009) Acid sphingomyelinase is a key regulator of cytotoxic granule secretion by primary T lymphocytes. Nat Immunol 10:761–768
38. Holopainen JM, Angelova MI, Kinnunen PK (2000) Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes. Biophys J 78:830–838
39. Idone V, Tam C, Goss JW, Toomre D, Pypaert M, Andrews NW (2008) Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis. J Cell Biol 180:905–914
40. Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, Hunter CA, Wherry EJ, Lindsten T, Reiner SL (2008) Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science 321:408–411
41. Jordan MB, Hildeman D, Kappler J, Marrack P (2004) An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood 104:735–743
42. Kagi D, Ledermann B, Burki K, Hengartner H, Zinkernagel RM (1994) CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity. Eur J Immunol 24:3068–3072

43. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H (1994) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369:31–37
44. Kataoka T, Togashi K, Takayama H, Takaku K, Nagai K (1997) Inactivation and proteolytic degradation of perforin within lytic granules upon neutralization of acidic pH. Immunology 91:493–500
45. Keefe D, Shi L, Feske S, Massol R, Navarro F, Kirchhausen T, Lieberman J (2005) Perforin triggers a plasma membrane-repair response that facilitates CTL induction of apoptosis. Immunity 23:249–262
46. Krzewski K, Coligan JE (2012) Human NK cell lytic granules and regulation of their exocytosis. Front Immunol 3:335
47. Kurschus FC, Bruno R, Fellows E, Falk CS, Jenne DE (2005) Membrane receptors are not required to deliver granzyme B during killer cell attack. Blood 105:2049–2058
48. Law RH, Lukoyanova N, Voskoboinik I, Caradoc-Davies TT, Baran K, Dunstone MA, D'Angelo ME, Orlova EV, Coulibaly F, Verschoor S, Browne KA, Ciccone A, Kuiper MJ, Bird PI, Trapani JA, Saibil HR, Whisstock JC (2010) The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature 468:447–451
49. Lichtenheld MG, Olsen KJ, Lu P, Lowrey DM, Hameed A, Hengartner H, Podack ER (1988) Structure and function of human perforin. Nature 335:448–451
50. Lichtenheld MG, Podack ER (1992) Structure and function of the murine perforin promoter and upstream region. Reciprocal gene activation or silencing in perforin positive and negative cells. J Immunol 149:2619–2626
51. Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO (2009) Integrin-dependent organization and bidirectional vesicular traffic at cytotoxic immune synapses. Immunity 31:99–109
52. Lopez JA, Susanto O, Jenkins MR, Lukoyanova N, Sutton VR, Law RH, Johnston A, Bird CH, Bird PI, Whisstock JC, Trapani JA, Saibil HR, Voskoboinik I (2013) Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. Blood 121:2659–2668
53. Masson D, Peters PJ, Geuze HJ, Borst J, Tschopp J (1990) Interaction of chondroitin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. Biochemistry 29:11229–11235
54. McCormack R, de Armas LR, Shiratsuchi M, Ramos JE, Podack ER (2013) Inhibition of intracellular bacterial replication in fibroblasts is dependent on the perforin-like protein (perforin-2) encoded by macrophage-expressed gene 1. J Innate Immun 5:185–194
55. McNeil PL, Kirchhausen T (2005) An emergency response team for membrane repair. Nat Rev Mol Cell Biol 6:499–505
56. Mehta PA, Davies SM, Kumar A, Devidas M, Lee S, Zamzow T, Elliott J, Villanueva J, Pullen J, Zewge Y, Filipovich A (2006) Perforin polymorphism A91 V and susceptibility to B-precursor childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Leukemia 20:1539–1541
57. Metkar SS, Wang B, Aguilar-Santelises M, Raja SM, Uhlin-Hansen L, Podack E, Trapani JA, Froelich CJ (2002) Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. Immunity 16:417–428
58. Metkar SS, Wang B, Catalan E, Anderluh G, Gilbert RJ, Pardo J, Froelich CJ (2011) Perforin rapidly induces plasma membrane phospholipid flip-flop. PLoS ONE 6:e24286
59. Millard PJ, Henkart MP, Reynolds CW, Henkart PA (1984) Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. J Immunol 132:3197–3204
60. Motyka B, Korbutt G, Pinkoski MJ, Heibein JA, Caputo A, Hobman M, Barry M, Shostak I, Sawchuk T, Holmes CF, Gauldie J, Bleackley RC (2000) Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. Cell 103:491–500

61. Mullbacher A, Ebnet K, Blanden RV, Hla RT, Stehle T, Museteanu C, Simon MM (1996) Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia. Proc Natl Acad Sci USA 93:5783–5787
62. Northrop JK, Wells AD, Shen H (2008) Cutting edge: chromatin remodeling as a molecular basis for the enhanced functionality of memory CD8 T cells. J Immunol 181:865–868
63. Ohkawa T, Seki S, Dobashi H, Koike Y, Habu Y, Ami K, Hiraide H, Sekine I (2001) Systematic characterization of human CD8+ T cells with natural killer cell markers in comparison with natural killer cells and normal CD8+ T cells. Immunology 103:281–290
64. Orange JS (2007) The lytic NK cell immunological synapse and sequential steps in its formation. Adv Exp Med Biol 601:225–233
65. Pachlopnik Schmid J, Cote M, Menager MM, Burgess A, Nehme N, Menasche G, Fischer A, de Saint Basile G (2010) Inherited defects in lymphocyte cytotoxic activity. Immunol Rev 235:10–23
66. Pachlopnik Schmid J, Ho CH, Chretien F, Lefebvre JM, Pivert G, Kosco-Vilbois M, Ferlin W, Geissmann F, Fischer A, de Saint Basile G (2009) Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. EMBO Mol Med 1:112–124
67. Pearce EL, Mullen AC, Martins GA, Krawczyk CM, Hutchins AS, Zediak VP, Banica M, DiCioccio CB, Gross DA, Mao CA, Shen H, Cereb N, Yang SY, Lindsten T, Rossant J, Hunter CA, Reiner SL (2003) Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science 302:1041–1043
68. Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, Slot JW, Geuze HJ (1991) Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 173:1099–1109
69. Pipkin ME, Ljutic B, Cruz-Guilloty F, Nouzova M, Rao A, Zuniga-Pflucker JC, Lichtenheld MG (2007) Chromosome transfer activates and delineates a locus control region for perforin. Immunity 26:29–41
70. Pipkin ME, Rao A, Lichtenheld MG (2010) The transcriptional control of the perforin locus. Immunol Rev 235:55–72
71. Podack ER, Young JD, Cohn ZA (1985) Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. Proc Natl Acad Sci USA 82:8629–8633
72. Praper T, Sonnen A, Viero G, Kladnik A, Froelich CJ, Anderluh G, Dalla Serra M, Gilbert RJ (2011) Human perforin employs different avenues to damage membranes. J Biol Chem 286:2946–2955
73. Praper T, Sonnen AF, Kladnik A, Andrighetti AO, Viero G, Morris KJ, Volpi E, Lunelli L, Dalla Serra M, Froelich CJ, Gilbert RJ, Anderluh G (2011) Perforin activity at membranes leads to invaginations and vesicle formation. Proc Natl Acad Sci USA 108:21016–21021
74. Ramachandran R, Heuck AP, Tweten RK, Johnson AE (2002) Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. Nat Struct Biol 9:823–827
75. Ramachandran R, Tweten RK, Johnson AE (2004) Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment. Nat Struct Mol Biol 11:697–705
76. Reddy A, Caler EV, Andrews NW (2001) Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell 106:157–169
77. Rosado CJ, Buckle AM, Law RH, Butcher RE, Kan WT, Bird CH, Ung K, Browne KA, Baran K, Bashtannyk-Puhalovich TA, Faux NG, Wong W, Porter CJ, Pike RN, Ellisdon AM, Pearce MC, Bottomley SP, Emsley J, Smith AI, Rossjohn J, Hartland EL, Voskoboinik I, Trapani JA, Bird PI, Dunstone MA, Whisstock JC (2007) A common fold mediates vertebrate defense and bacterial attack. Science 317:1548–1551
78. Rutishauser RL, Kaech SM (2010) Generating diversity: transcriptional regulation of effector and memory CD8 T-cell differentiation. Immunol Rev 235:219–233

79. Salcedo TW, Azzoni L, Wolf SF, Perussia B (1993) Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. J Immunol 151:2511–2520
80. Santoro A, Cannella S, Trizzino A, Lo Nigro L, Corsello G, Arico M (2005) A single amino acid change A91 V in perforin: a novel, frequent predisposing factor to childhood acute lymphoblastic leukemia? Haematologica 90:697–698
81. Sauer H, Pratsch L, Tschopp J, Bhakdi S, Peters R (1991) Functional size of complement and perforin pores compared by confocal laser scanning microscopy and fluorescence microphotolysis. Biochim Biophys Acta 1063:137–146
82. Schreiber TH, Podack ER (2009) A critical analysis of the tumour immunosurveillance controversy for 3-MCA-induced sarcomas. Br J Cancer 101:381–386
83. Shepard LA, Heuck AP, Hamman BD, Rossjohn J, Parker MW, Ryan KR, Johnson AE, Tweten RK (1998) Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin Clostridium perfringens perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. Biochemistry 37:14563–14574
84. Shi L, Keefe D, Durand E, Feng H, Zhang D, Lieberman J (2005) Granzyme B binds to target cells mostly by charge and must be added at the same time as perforin to trigger apoptosis. J Immunol 174:5456–5461
85. Shi L, Mai S, Israels S, Browne K, Trapani JA, Greenberg AH (1997) Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. J Exp Med 185:855–866
86. Shinkai Y, Takio K, Okumura K (1988) Homology of perforin to the ninth component of complement (C9). Nature 334:525–527
87. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA (2000) Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. J Exp Med 192:755–760
88. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V (1999) Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science 286:1957–1959
89. Street SE, Zerafa N, Iezzi M, Westwood JA, Stagg J, Musiani P, Smyth MJ (2007) Host perforin reduces tumor number but does not increase survival in oncogene-driven mammary adenocarcinoma. Cancer Res 67:5454–5460
90. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100:655–669
91. Tam C, Idone V, Devlin C, Fernandes MC, Flannery A, He X, Schuchman E, Tabas I, Andrews NW (2010) Exocytosis of acid sphingomyelinase by wounded cells promotes endocytosis and plasma membrane repair. J Cell Biol 189:1027–1038
92. Terasaki M, Miyake K, McNeil PL (1997) Large plasma membrane disruptions are rapidly resealed by Ca2+-dependent vesicle-vesicle fusion events. J Cell Biol 139:63–74
93. Thiery J, Keefe D, Boulant S, Boucrot E, Walch M, Martinvalet D, Goping IS, Bleackley RC, Kirchhausen T, Lieberman J (2011) Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. Nat Immunol 12:770–777
94. Thiery J, Keefe D, Saffarian S, Martinvalet D, Walch M, Boucrot E, Kirchhausen T, Lieberman J (2010) Perforin activates clathrin- and dynamin-dependent endocytosis, which is required for plasma membrane repair and delivery of granzyme B for granzyme-mediated apoptosis. Blood 115:1582–1593
95. Trambas C, Gallo F, Pende D, Marcenaro S, Moretta L, De Fusco C, Santoro A, Notarangelo L, Arico M, Griffiths GM (2005) A single amino acid change, A91 V, leads to conformational changes that can impair processing to the active form of perforin. Blood 106:932–937
96. Trapani JA, Sutton VR, Thia KY, Li YQ, Froelich CJ, Jans DA, Sandrin MS, Browne KA (2003) A clathrin/dynamin- and mannose-6-phosphate receptor-independent pathway for granzyme B-induced cell death. J Cell Biol 160:223–233

97. Trimble LA, Lieberman J (1998) Circulating CD8 T lymphocytes in human immunodeficiency virus-infected individuals have impaired function and downmodulate CD3 zeta, the signaling chain of the T-cell receptor complex. Blood 91:585–594
98. Tschopp J, Masson D, Stanley KK (1986) Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. Nature 322:831–834
99. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM (1997) Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. EMBO J 16:7287–7296
100. Vivier E, Sorrell JM, Ackerly M, Robertson MJ, Rasmussen RA, Levine H, Anderson P (1993) Developmental regulation of a mucinlike glycoprotein selectively expressed on natural killer cells. J Exp Med 178:2023–2033
101. Voskoboinik I, Dunstone MA, Baran K, Whisstock JC, Trapani JA (2010) Perforin: structure, function, and role in human immunopathology. Immunol Rev 235:35–54
102. Voskoboinik I, Smyth MJ, Trapani JA (2006) Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol 6:940–952
103. Voskoboinik I, Sutton VR, Ciccone A, House CM, Chia J, Darcy PK, Yagita H, Trapani JA (2007) Perforin activity and immune homeostasis: the common A91 V polymorphism in perforin results in both presynaptic and postsynaptic defects in function. Blood 110:1184–1190
104. Voskoboinik I, Thia MC, De Bono A, Browne K, Cretney E, Jackson JT, Darcy PK, Jane SM, Smyth MJ, Trapani JA (2004) The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. J Exp Med 200:811–816
105. Voskoboinik I, Thia MC, Fletcher J, Ciccone A, Browne K, Smyth MJ, Trapani JA (2005) Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: a critical role for aspartate residues 429, 435, 483, and 485 but not 491. J Biol Chem 280:8426–8434
106. Walsh CM, Matloubian M, Liu CC, Ueda R, Kurahara CG, Christensen JL, Huang MT, Young JD, Ahmed R, Clark WR (1994) Immune function in mice lacking the perforin gene. Proc Natl Acad Sci USA 91:10854–10858
107. Werneck MB, Lugo-Villarino G, Hwang ES, Cantor H, Glimcher LH (2008) T-bet plays a key role in NK-mediated control of melanoma metastatic disease. J Immunol 180:8004–8010
108. Wherry EJ (2011) T cell exhaustion. Nat Immunol 12:492–499
109. Yamamoto K, Shibata F, Miyasaka N, Miura O (2002) The human perforin gene is a direct target of STAT4 activated by IL-12 in NK cells. Biochem Biophys Res Commun 297:1245–1252
110. Youn BS, Kim KK, Kwon BS (1996) A critical role of Sp1- and Ets-related transcription factors in maintaining CTL-specific expression of the mouse perforin gene. J Immunol 157:3499–3509
111. Yu CR, Ortaldo JR, Curiel RE, Young HA, Anderson SK, Gosselin P (1999) Role of a STAT binding site in the regulation of the human perforin promoter. J Immunol 162:2785–2790
112. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R (1998) Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 188:2205–2213
113. Zediak VP, Wherry EJ, Berger SL (2011) The contribution of epigenetic memory to immunologic memory. Curr Opin Genet Dev 21:154–159
114. Zhang J, Scordi I, Smyth MJ, Lichtenheld MG (1999) Interleukin 2 receptor signaling regulates the perforin gene through signal transducer and activator of transcription (Stat)5 activation of two enhancers. J Exp Med 190:1297–1308

115. Zhang Y, Lichtenheld MG (1997) Non-killer cell-specific transcription factors silence the perforin promoter. J Immunol 158:1734–1741
116. Zhou J, Zhang J, Lichtenheld MG, Meadows GG (2002) A role for NF-kappa B activation in perforin expression of NK cells upon IL-2 receptor signaling. J Immunol 169:1319–1325
117. Zhu Y, Ju S, Chen E, Dai S, Li C, Morel P, Liu L, Zhang X, Lu B (2010) T-bet and eomesodermin are required for T cell-mediated antitumor immune responses. J Immunol 185:3174–3183