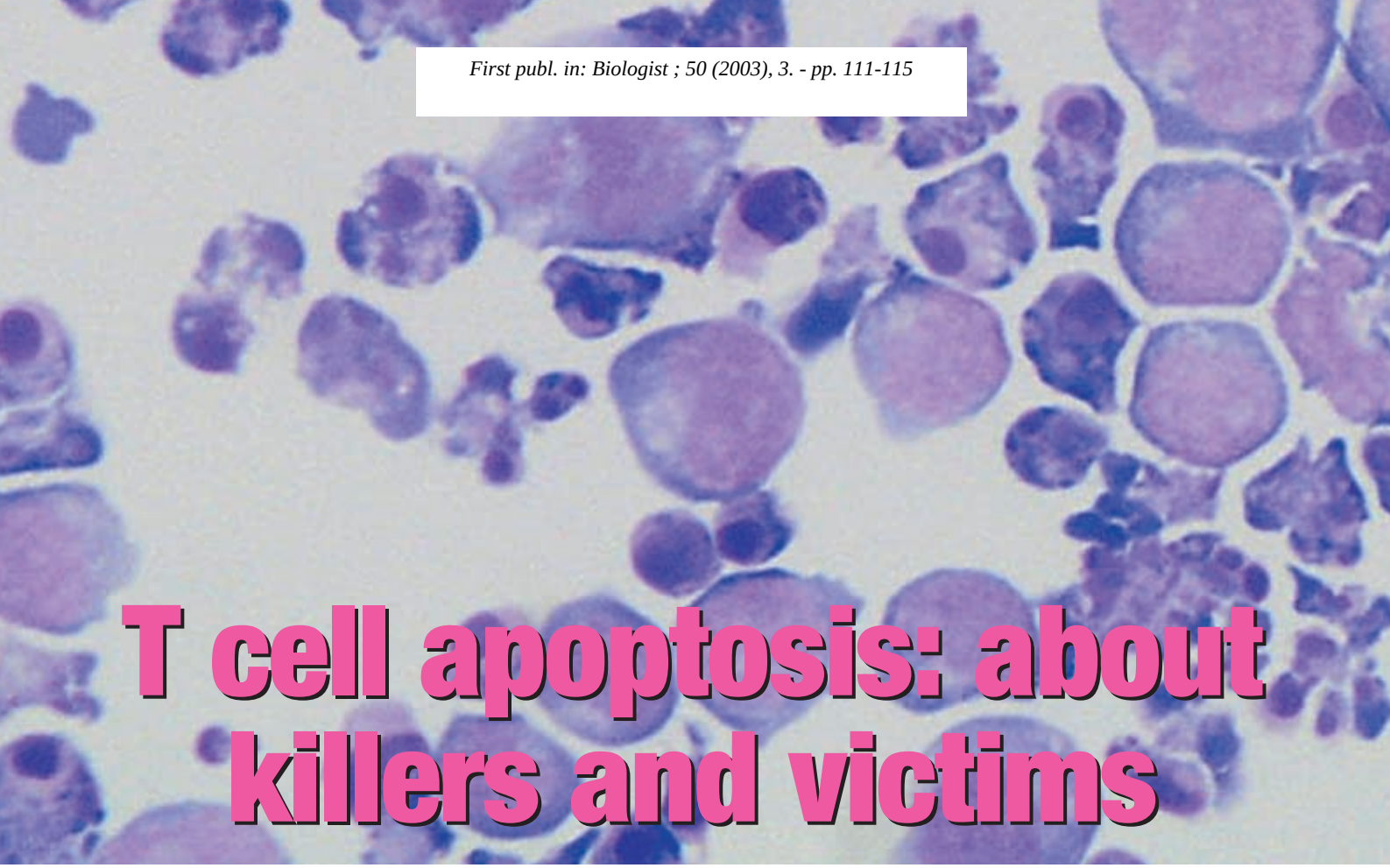
First publ. in: Biologist ; 50 (2003), 3. - pp. 111-115



T cell apoptosis: about killers and victims

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For multicellular organisms, such as ourselves, to function properly, it is just as important for certain cells to die when not needed as it is for others to survive. Nowhere is apoptosis more crucial to proper function than in the immune system.

T lymphocytes are important regulatory cells that help to orchestrate our immune system to defend our body from harmful pathogens. However, not all T cell-mediated immune responses are beneficial. Uncontrolled T cell activation and expansion is the underlying cause of many destructive diseases. The more we understand about these diseases and their regulation, the more it becomes evident that many defects originate in the (in)ability of T cells to die. This review will discuss two important aspects of cell death in the regulation of T cell functions: the ability to commit suicide and the ability to kill.

Cell growth and cell death: a balancing act

The potential of stem cells to divide and differentiate into different tissue cells is the basis of forming complex multicellular organisms. While cell division and growth is a prerequisite to an increase in cell number and cell mass, recent evidence supports the idea that cell death is equally important for the controlled growth of a body. Every cell in our body contains the genetic program for cell death, and this program is as important as the program to multiply and differentiate. During embryonic development, cell death is an integral part of the developmental program. The capacity of individual cells to die permits every anatomical structure within the fetus to be remodelled, and makes sure that organs, bones and limbs are properly shaped. For example, the limbs are first built as paddles and specific induction of cell death in the tissue between the digits leads to their separation and the formation of functional fingers and toes. Thus, cell death sculpts body tissue like a hammer and chisel sculpt marble to form a statue.

In the adult body, tissue growth and sculpting is minimal, and the role of cell death is shifted towards maintenance of cellular homeostasis and turnover. It ensures that old, damaged or undesired cells are efficiently removed. The importance of this homeostatic process is illustrated by the fact that every minute our body produces about five million new cells and about the same quantity must die by cellular suicide. Deregulation of this dynamic equilibrium often results in severe disease, such as cancer and autoimmune diseases (too little cell death), or neurodegenerative diseases, such as Alzheimer's Disease (too much cell death).

Roads to ruin: pathways leading to apoptosis

The role of cell death in development and cellular homeostasis has started to be understood only in the last twenty years (and much more remains to be discovered). However, the initial idea that cell death may represent an important mechanism for the maintenance of cellular homeostasis was already recognised by the German anatomist Ludwig Gräper in 1915. He postulated that the capacity of cells to die by 'chromatolysis' is an important feature present in

every organ to counterbalance mitosis. The term chromatolysis was given in 1885 by the anatomist Walter Flemming to describe a phenomenon that, almost 100 years later, would be called apoptosis! Gräper 's observation however, passed quite unnoticed until the fifties, when it was finally recognised that cells can commit suicide and that this suicide program is imprinted in every single cell. Several years later, at the beginning of the seventies, Kerr, Wyllie and Currie observed that some dying cells showed characteristic morphological changes that were distinct from those occurring during accidental death by necrosis. This phenomenon was called '*apoptosis*' (Kerr *et al*., 1972), literally meaning 'falling off', and has been compared with natural process of leaves falling off the tree in autumn.

Apoptotic cell death proceeds via many different, distinct phases and this further underlines the active nature of apoptosis compared to the passive cell death by necrosis. While necrotic cell death can be considered an 'accident', apoptosis is an energy-dependent process, which is actively initiated. A characteristic feature of apoptotic cells is cellular and nuclear condensation. Quite often, however, fragmentation of the nucleus and the cell into small membrane-associated vesicles (so-called apoptotic bodies) is observed (Figure 1). These typical morphological features associated with cells dying by apoptosis are the result of defined and evolutionarily conserved biochemical pathways.

Apoptosis can be induced by an enormous variety of stimuli. However, different cell types display differential sensitivity towards apoptosis induction. Since apoptotic cells have common morphological characteristics, it is evident that common biochemical pathways must exist in most cell types and that these pathways are activated by all apoptosis-inducing triggers. Central to most, if not all, forms of apoptotic cell death is the activation of caspases. Caspases are a group of proteases that contain a cystein in their active site and preferentially cleave their substrates after aspartic acid (*C*ystein-*Asp*artate Prote*ases*). Caspases are always present in their inactive pro-form. Apoptosis induction leads to proteolytic cleavage of the pro-form and assembly of an enzymatically active mature caspase, composed of two small and two large subunits. Importantly, caspase inhibition, *e.g.*, via specific inhibitors, blocks almost all forms of apoptosis (Figure 1). But how are caspases involved in apoptotic cell death?

Caspase activity can lead to proteolytic digestion of target proteins and thus to their inactivation. For example, many structural cell proteins are caspase substrates, and their cleavage leads to their disassembly and the typical morphological alterations observed during apoptosis. Caspases can also cleave and inactivate many proteins involved in gene transcription and other signalling pathways, thus inhibiting certain processes that are not required by the dying cell, or that must be terminated. The proteolytic digestion of these substrates and their inactivation represents the actual 'demolition phase' of apoptotic cell death. However, caspases do not only inactivate proteins, but may also activate enzymatic processes. Caspases can cleave their own or other caspases' pro-forms, resulting in further amplification and induction of an enzymatic cascade. The most prominent caspase-dependent activation process is that of the nuclease responsible for digestion of the genome, a hallmark in the apoptosis process. Caspases cleave and inactivate a specific nuclease inhibitor, releasing the active enzyme, which digests nuclear DNA and prevents further gene transcription (Hengartner, 2000).

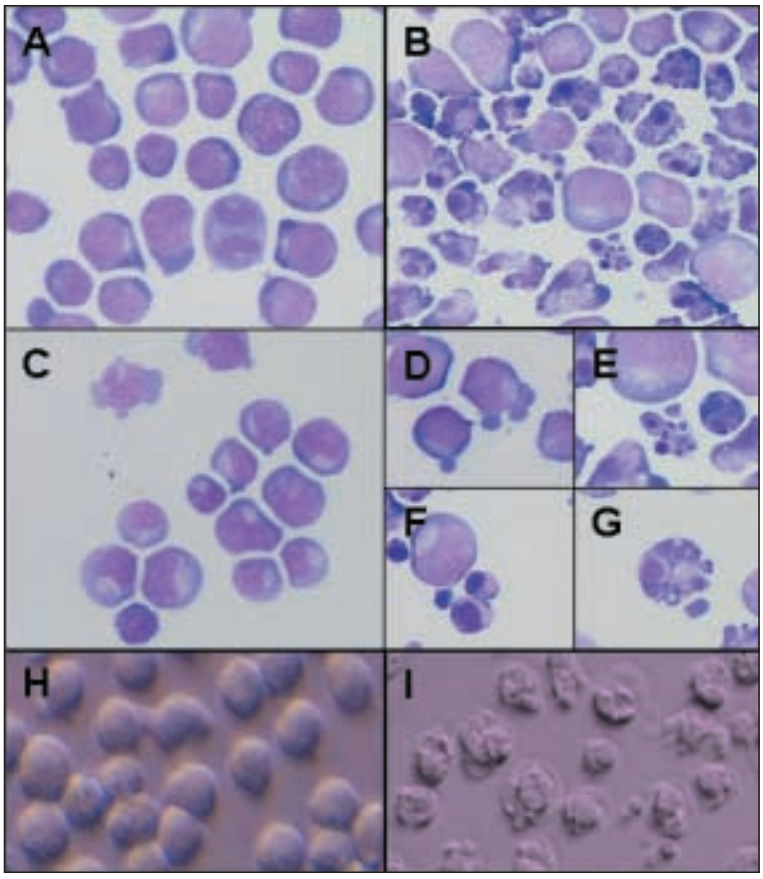

Figure 1. Morphological changes in cells undergoing apoptosis. *The leukaemic T cell line Jurkat has been treated with UV irradiation to undergo apoptosis. A) Normal morphology of live cells. B) Different apoptotic morphologies upon UV irradiation. C) Caspase activation is required for apoptotic cell death. UV irradiated cells show normal morphology when caspases are blocked by the synthetic inhibitor zVAD-fmk. D) Membrane blebs in early apoptotic cells. E) Fragmentation of the apoptotic cell (apoptotic bodies). F) Cellular and nuclear condensation. G) Nuclear fragmentation. H) Phase contrast pictures of live and I) apoptotic cells.*

Although the caspase activation step is common to most apoptotic pathways, there are differences in signalling events initiating caspase activation depending whether the apoptosis-inducing stimulus is initiated 'inside' or 'outside' the cell. Oncogenes, hypoxia, cellular stress and DNA damaging agents (such as γ and UV irradiation, and chemotherapeutics) act inside cells and are sensed by molecules like that encoded by the tumour suppressor gene, p53. p53 activates pro-apoptotic members of the Bcl-2 family, which create pores in the outer membranes of mitochondria. As a result, cytochrome C is released into the cytoplasm, where it acts as a co-factor for assembly of the so-called apoptosome, consisting of multimers of the apoptosis-associated factor-1 (Apaf-1) and pro-caspase 9. Apoptosome assembly causes activation of caspase 9 and subsequently the caspase cascade. This pathway, since it is initiated inside the cell, is called the *intrinsic* pathway. In contrast, apoptosis induced via transmembrane receptors is transduced via the *extrinsic* pathway. Certain members of the tumour necrosis factor (TNF) receptor family, such as TNF receptor 1, Fas (CD95) and TRAIL receptors 1 and 2, contain distinct signalling motifs in their cytoplasmic tail, so called *death domains*, which are required for apoptosis induction. Thus, these receptors are

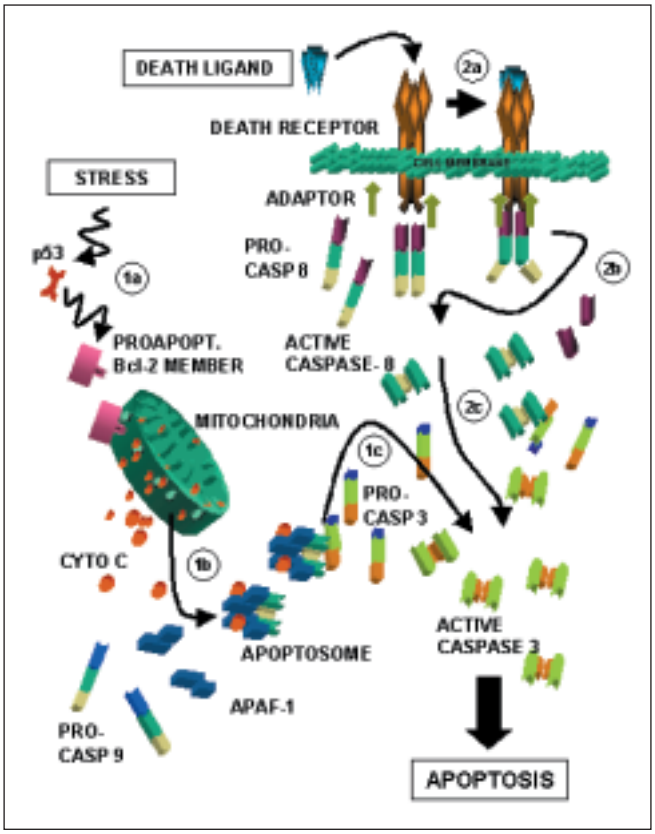

Figure 2. The two major apoptosis pathways. *The intrinsic pathway: cellular stress is sensed by the cell* (e.g. *by p53) leading to activation of pro-apoptotic members of the Bcl-2 family* (e.g. *Bax) (1a). This results in permeabilization of the outer mitochondrial membrane and release of cytochrome C (1b). Cytochrome C acts a co-factor for the formation of the apoptosome (Apaf-1 and caspase 9), which initiates the activation of the caspase cascade (1c). The extrinsic pathway: apoptosis is initiated by binding of death ligands to their respective death receptors* (e.g. *Fas) (2a). Binding induces trimerization and a conformational change. This leads to recruitment of adaptor molecules and pro-caspase 8, which becomes proteolytically cleaved and activated (2b). Active caspase 8 initiates the caspase cascade by activating other caspases (2c). At this level the intrinsic and extrinsic pathway proceed in common. Caspase activation leads to the apoptotic execution of the cell.*

called *death receptors*. Ligand binding causes receptor trimerisation, and subsequent conformational changes trigger the recruitment of adaptor molecules and pro-forms of caspases, which subsequently become activated. At this level of caspase activation the intrinsic and the extrinsic pathways merge and proceed together (Figure 2) (Hengartner, 2000; Strasser, 2000).

T cell suicide and immune homeostasis

The immune system that defends our body from infectious agents follows a simple principle: expand on need, reduce when done. Under conditions of bacterial or viral infection, numbers of leucocytes participating in the immune response dramatically increase, gradually declining when the infection resolves. This phenomenon is particularly well pronounced in antigen-specific T lymphocytes. T cells recognise antigen bound to the major histocompatibility complex (MHC, the antigen-presenting structure) via a specific receptor (T cell receptor). This leads to activation of the cell, proliferation and the production of many cytokines (soluble protein mediators) that enhance and orchestrate immune responses. For example, specific T cell activation and cytokine production is required for the production of neutralising antibodies by B lymphocytes. Similarly, activated T cells can recognise virus-infected cells and kill these cells, as we will see later, also by apoptosis induction.

Recent years have led to the development of technologies that allow us to follow the fate of antigen-specific T lymphocytes during immune responses. Usually, the frequency of T cells specific to an individual antigen is rather low since the entire T lymphocyte repertoire must be ready to combat not only a single pathogen but an enormous variety of different antigens. Thus, upon encounter with the specific antigen, *e.g.*, during a viral infection, the few antigen-specific T cells must expand dramatically to reach a critical number and efficiently eliminate the virus. Experiments in mice have shown that, under these conditions, virus-specific T lymphocytes can easily make up to 20% of all T cells. It is obvious that this enormous expansion has to be followed by a radical depletion phase. And not surprisingly, upon resolution of the infection, the number of antigen-specific T cells usually drops dramatically. This process is called *peripheral deletion* and involves apoptotic cell death of the antigen-specific T cells.

There are two major mechanisms through which T cell homeostasis is induced by apoptosis: a passive mechanism, via 'death-by-neglect' and an active mechanism, via 'activation-induced cell death'. Growing T cells are dependent on growth factors. Many of these growth factors are produced by the activated T cells themselves, *e.g.*, interleukin 2, and act in an autocrine manner. Once the pathogen has been eliminated, T cell activation is reduced and less growth factors are produced. Since growth factors are also survival factors for activated T cells, a lack of survival signals sensitises them for apoptosis induction via the intrinsic pathway. This passive form of T cell apoptosis is called *death-by-neglect* (Lenardo *et al.*, 1999). While the actual trigger of apoptosis is unknown, it is believed that growth factor signals antagonise this process through induction of different anti-apoptotic signals.

Growth factor levels only gradually decrease however, and immune homeostasis mediated by death-by-neglect must be considered a relatively slow process. Additional, faster mechanisms must exist to mediate the relatively rapid T cell depletion observed *in vivo*. Interestingly, while stimulation of resting T cells induces proliferation and effector functions, restimulation of pre-activated T cell causes rapid induction of apoptosis. But what is the underlying mechanism of this *activation-induced cell death?* Important clues came from two natural mutant mouse strains, the *lpr* (*l*ympho*pr*oliferative disorder) and *gld* (*g*eneralized *l*ymphoproliferative *d*isorder) mice. With advancing age, these mice accumulate activated T and B lymphocytes and often develop autoimmune diseases. Cloning of the defective genes revealed that *lpr* encodes the apoptosis-inducing death receptor Fas (CD95) and *gld* encodes its ligand. Stimulated T cells express both the Fas receptor and its ligand, and it appears likely that they can interact with each other and induce apoptosis. Indeed, activation-induced cell death in T cells is efficiently blocked by neutralising antibodies against Fas or Fas ligand (see Figure 3). Thus, restimulation of pre-activated T cells induces the simultaneous expression of Fas and Fas ligand and subsequently Fas-mediated apoptosis via the extrinsic pathway (Brunner *et al.*, 1995). Interestingly, this process even occurs in a single cell and is thus cell-autonomous (suicide), although fratricide killing of neighbouring T cells may also be involved. Similarly, activation-induced cell death proceeds not only via Fas-Fas ligand interactions, but other members of the TNF family (*e.g.*, TNFα and TRAIL) have also been implicated (Lenardo *et al.*, 1999).

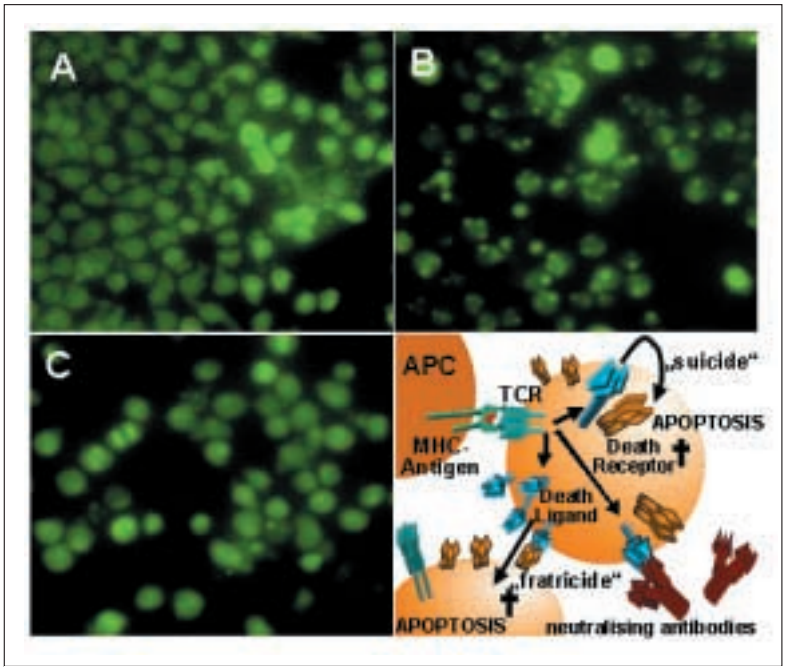

Figure 3. Activation-induced cell death in T cells. *T cells were stained with acridine orange which binds nucleic acids and appears green when viewed by fluorescence microscopy. A) Normal nuclear morphology in live cells. B) Apoptotic nuclear morphology in cells undergoing activation-induced cell death. C) Normal nuclear morphology in cells where activation-induced cell death has been blocked with anti-Fas ligand antibody. D) Proposed mechanism of activation-induced cell death in T cells: T cell receptor activation induces the expression of Fas and Fas ligand and subsequent cell-autonomous (suicide) interaction or interaction with neighbouring cells (fratricide) induces apoptosis via the extrinsic death receptor-dependent pathway.*

T cell cytotoxicity: murder by persuasion (an offer that cannot be refused…)

If T cells can commit Fas ligand-dependent suicide, they can probably also kill other cells. Indeed, death ligand-induced apoptosis is one of the major mechanisms by which cytotoxic T cells kill their target cells. In particular, activated $CD8^+$ cytotoxic T cells rapidly express Fas ligand upon recognition of antigen on target cells, and subsequent interaction with Fas 'persuades' the target cell to undergo apoptotic cell death. In $CD4^+$ T helper cells, Fas ligand-mediated cytotoxicity is even the dominant mechanism of target cell killing. The importance of this cytotoxic effector mechanism is nicely illustrated during hepatitis. In this condition, liver cells are predominantly killed by Fas ligand expressed on infiltrating cytotoxic T cells, and inhibition of Fas ligand abrogates tissue destruction. Fas ligand transcription is a relatively slow process. To speed up Fas ligand-mediated cytotoxicity, T cells have developed an interesting strategy. Upon transcription, Fas ligand is not only transported to the cell membrane, but is also stored in cytoplasmic granule-like vesicles. Activation of the T cell by the target cell induces the rapid release of Fas ligand to the cell surface, making it more readily available to induce apoptosis in the target cell (Bossi and Griffiths, 1999; Wasem, 2001).

The degranulation of previously synthesised and stored cytotoxic Fas ligand molecules strongly resembles the other dominant mechanism of cell-mediated cytotoxicity. Activated $CD8^+$ T cells and natural killer cells (NK cells) express the pore-forming protein perforin and the serine protease granzyme B, which are stored in so-called cytotoxic granules. Specific stimulation of cytotoxic T cells by antigen on target cells induces the release of these proteins into the extracellular space, sometimes referred to as 'synapse', between T cell and target cell. Whereas granzyme B binds to the target cell surface via a specific receptor (manose-6-phosphate receptor), it cannot enter the cell without the help of *perforin*. In the presence of calcium, perforin polymerises on the cell membrane and allows *granzyme B* to be released into the cytoplasm. Cytoplasmic granzyme B cleaves a variety of target proteins, but most importantly, it cleaves and activates caspases, and initiates the apoptosis cascade.

Although these two major ways of target cell killing involve different effector molecules, they have similar strategies in common: the rapid release of preformed effector molecules, and the activation of the cell's own apoptosis machinery. Thus, cytotoxic T cells *persuade* their target cells to commit suicide (Froelich *et al*., 1998). The question remains as to why it makes sense that target cells undergo apoptosis rather than just being lysed. The answer may well lie in the completely different effects that necrotic and apoptotic cell death have on the immune system. Necrosis leads to cell disruption and the release of the entire cellular content. Interestingly, many of these components are immunostimulatory. Thus, necrotic cell death always causes inflammation and further tissue destruction. In contrast, apoptotic cell death proceeds quietly, since apoptotic cells are engulfed by phagocytes while their membranes are still intact. The immune system appears to prefer this quiet, clean and 'non-spreading' mechanism to specifically eliminate target cells over the contagious cell death by necrosis.

Too little, too much….

T cell apoptosis is a tightly regulated process. Appropriately expressed apoptosis-inducing molecules, such as

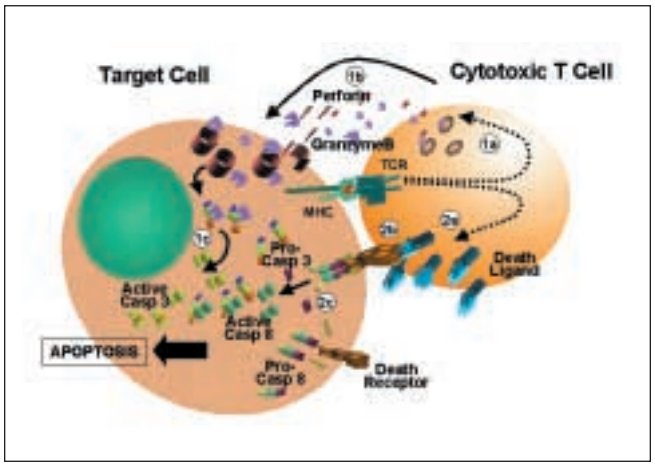

Figure 4. The two major mechanisms of T cell-mediated cytotoxicity. *T cells recognise antigen via their T cell receptor (TCR). This leads to the degranulation of cytotoxic granules (1a), and the release of perforin and granzyme B (1b). Perforin polymerises on the target cell membrane and allows granzyme B to translocate to the cytoplasm of the target cell. There it cleaves and activates caspase 3 and initiates the apoptosis pathway (1c). T cell activation also leads to the expression of death ligands (*e.g. *Fas ligand) (2a), which binds to death receptors on the target cell (2b). Upon adaptor molecule recruitment and caspase 8 activation, the caspase cascade and apoptosis is initiated (2c).*

Fas and Fas ligand, and inhibitors of the intrinsic and extrinsic apoptosis pathways define the optimal time for the demise of the cell. This must not be too early, so that the T cell can complete the important task of immune regulation, and not too late, or it may become dangerous for the host. The importance of this perfect timing is nicely demonstrated in a variety of diseases that show abnormal T cell apoptosis. The human immunodeficiency virus (HIV) not only infects $CD4^+$ T cells for its replication, but it also induces apoptosis in these cells. As a result, levels of $CD4^+$ T helper cells dramatically fall. After normally harmless opportunistic infections, the immune system in these patients can no longer activate and orchestrate a protective immune response, and the HIV-infected patients become immunodeficient.

In marked contrast, in some inflammatory diseases, T lymphocytes show aberrantly high expression of anti-apoptotic molecules. As a consequence, antigen-specific T cells persist and continue to produce pro-inflammatory mediators that activate other immune cells. The result of this uncontrolled inflammation is severe tissue damage. The consequences of defective T cell apoptosis are particularly pronounced in patients with genetic defects in the key molecules of activation-induced cell death. Both mutations in Fas and the Fas ligand cause massive lymphoproliferative disorders. Most importantly, autoreactive T and B cells are no longer eliminated and, with increasing age, these patients suffer from severe autoimmune diseases and resulting tissue destruction (Lenardo *et al.*, 1999).

Cytotoxicity is meant to eliminate undesired cells, *e.g.*, virus-infected cells. However, sometimes the induction of apoptosis in target cells induces more damage to the infected organ than the virus infection alone would cause. The example of the T cell-induced damage during hepatitis has been mentioned already above. Similarly, rejection of a recently transplanted kidney, heart, or liver by cytotoxic T cells is a non-desired complication and is actively treated by immunosupressive agents.

In conclusion, apoptotic cell death plays an important role in T cell homeostasis and cytotoxicity. It regulates the extent of T cell responses and permits an appropriate balance between benefit and damage. Too little or too much apoptosis in T cells is often the underlying cause of immunological diseases and is a major target for therapeutic intervention.

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Website on different aspects of apoptosis

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