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Memory and death in the immune system

Apoptosis refers to a form of death in which a cell initiates a suicide program and in which characteristic morphological alterations are observed in dying and dead cells. These changes include chromatin condensation, nucleolar disruption, cytoplasmic contraction, dilation of the endoplasmic reticulum, and membrane blebbing. This form of death is typically accompanied by the cleavage of DNA into a "ladder" of oligonucleosome length fragments. Apoptotic cells are systematically dismantled into bite-size packages that are recognized and disposed of by tissue macrophages without the concomitant activation of the innate immune system. This form of death is not associated with inflammation, and is also referred to as "programmed cell death" or "physiological cell death".

Apoptosis may be initiated in a variety of circumstances including, but not limited to, the induction of DNA damage, the activation of a stress response, the withdrawal of growth factors, or the triggering of specific signaling receptors. This type of cell death is of critical importance in the development of virtually every multicellular organism. Apoptotic death is repeatedly initiated during lymphoid ontogeny, during the process of negative selection, whenever lymphocytes fail to be positively selected, and during the contraction phase of immune responses. Cytolytic CD8+T cells, Natural Killer cells, and activated CD4+ T cells express specific molecules, including Perforin, Granzyme B, and Fas ligand, that are designed to assist in the execution of target cells by apoptosis.

There are two major pathways that lead to apoptosis, both of which culminate in a common death pathway. The mitochondrial or intrinsic pathway involves the induction, by a variety of stimuli, of specialized proteins that induce mitochondrial leakiness and thus activate the common death pathway. In the extrinsic "death receptor" pathway, the triggering of cell surface receptors of the TNFR family contributes to the enzymatic activation of the common death pathway.

The common death pathway depends on the activation of caspases

The induction of death is generally linked to the activation of a set of proteolytic enzymes that are called caspases (cysteine proteases that cleave proteins immediately after **aspartic acid** residues). The first such enzyme to be identified was the *C. elegans* cell death gene, Ced-3. Caspases exist as latent pro-enzymes that are activated in a cascade-like fashion following cleavage of a pro-piece that ends in an aspartyl residue. All caspases contain a QACRG motif in which the cysteine residue is a part of the active site. "Initiator" caspases of the mitochondrial pathway and of the death receptor pathway cleave and activate "executioner" caspases of the common death pathway. Executioner caspases in turn cleave specific substrates and thus contribute to the dismantling and packaging of the dying cell. The caspase that has been best characterized in the context of the common death pathway is caspase 3.

A number of substrates of caspase-3 have been identified. A particularly interesting substrate is the Caspase activated DNase (CAD) also known as DNA Fragmentation Factor or DFF. CAD is a latent nuclease that can cleave chromosomal DNA into nucleosome size fragments but is held in check by an inhibitor called ICAD (for Inhibitor of CAD). The nuclease activity of CAD is released by caspase-3 mediated cleavage of ICAD. Activated CAD is responsible in part for the fragmentation of DNA that is characteristic of apoptosis.

The mitochondrial pathway and proteins of the Bcl-2 family

The predominant mechanism of apoptosis in all metazoan cells involves the regulation of mitochondrial integrity by members of the Bcl-2 family. Bcl-2 was initially identified during the molecular characterization of a chromosomal translocation in patients with follicular lymphoma that brings the immunoglobulin heavy chain locus on chromosome 14 in apposition with the Bcl-2 gene on chromosome 18. Bcl-2 represents the vertebrate homolog of *Ced-9*, a *C. elegans* gene that inhibits apoptosis in nematodes. Like *Ced-9*, Bcl-2 is an inhibitor of the mitochondrial pathway of apoptosis.

Bcl-2 family proteins can be broadly divided into three categories. Multi-domain anti-apoptotic proteins such as Bcl-2 and Bcl X_L, typically contain four BH (Bcl-2 homology) domains and reside in the outer mitochondrial membrane where they contribute to mitochondrial stability. Multi-domain pro-apoptotic proteins such as Bax and Bak, resemble Bcl-2 in having multiple domains but these proteins, when activated, can disrupt the integrity of the

outer mitochondrial membrane. Bak and Bax can bind in the cytoplasm to a range of ligand-like pro-apoptotic proteins known as single-domain BH3-only proteins. This binding event causes the multi-domain proteins to change conformation, oligomerize, translocate to the outer mitochondrial membrane, and induce the release of a key mitochondrial inducers of apoptosis including cytochrome c, Smac/Diablo, and Omi/HtrA2.

A number of different stimuli induce distinct BH3-only proteins by transcriptional and post-transcriptional means. DNA damage contributes to the p53 mediated transcriptional induction of genes encoding BH3-only proteins called Puma and Noxa. BCR and TCR signals in developing B and T cells induce a BH3 only protein called Bim by a number of mechanisms, including post-translational modification by phosphorylation. Activation of caspase-8 by death receptors, as discussed below, can induce the activation of a latent BH3-only protein called Bid by proteolytic processing. Once an active form of a BH3-only protein is induced, it has the potential to collaborate with Bak and Bax to induce the loss of mitochondrial integrity. This potential may not be realized in the presence of an excess of anti-apoptotic proteins like Bcl-2 which function as a "sink" binding to the BH3-only proteins, and thus preventing the activation and oligomerization of Bak and Bax.

If an excess of a BH3 only protein is induced, cytochrome c released from mitochondria binds to a protein called APAF-1 (Apoptosis activating factor-1), which is the vertebrate homolog of Ced-4, a key regulator of apoptosis in the nematode *C.elegans*. The Apaf-1/cytochrome-c complex, also known as the apoptosome, binds to and activates procaspase-9, a key initiator caspase in the mitochondrial pathway. Caspase-9 in turn activates the common death pathway.

Apart from cytochrome c, other released mitochondrial proteins also contribute to caspase activation, but in a less direct manner. Activated caspases in the cytosol associate with and are held in check by a family of inhibitors, known as IAPs, (for inhibitors of apoptosis). Two mitochondrial proteins released during apoptosis, Smac/Diablo and Omi/HtrA2, bind to and antagonize the activity of the IAPs and thereby promote the maintenance of caspase activity and the induction of apoptosis.

The Death Receptor Pathway

A number of trimeric cell surface receptors of the TNFR family can induce apoptosis when they bind to their

physiological ligands. These receptors are generically referred to as death receptors, and the prototypic death receptor is Fas. Fas ligand (FasL) is induced in activated CD4+T cells, and its interaction with Fas contributes to the contraction of helper T cell responses. FasL on activated helper T cells also interacts with Fas on B cells to mediate the elimination of bystander B cells that have been non-specifically activated by CD40L (which is also expressed on activated T helper cells).

The intracellular portion of Fas contains a protein-protein interaction domain known as a death domain. A death domain-containing adaptor protein called FADD (Fas Associated Death Domain) links Fas to procaspase-8 on the inner face of the plasma membrane. Ligation of Fas by FasL leads to the activation of caspase-8 and consequently the induction of apoptosis.

Although death receptor pathways can theoretically induce apoptosis without requiring mitochondrial changes, death receptor signaling is frequently amplified by a positive feedback mechanism involving mitochondria. Caspase-8, in many cells, will cleave and activate a pro-apoptotic BH3-only protein called Bid. Activated Bid in turn contributes to the mitochondrial pathway via Bak and Bax as discussed above

The abrogation of apoptosis contributes to memory

There are a number of signaling events and molecules that protect cells from apoptosis. Many of these signals culminate in the activation of an anti-apoptotic member of the Bcl-2 family or the inactivation of a pro-apoptotic member of the same family. Early in T cell development activation of the IL-7 receptor provide survival signals possibly by influencing the levels of Bcl-2. Antigen and pre-antigen receptor signals can contribute to the up-regulation of another anti-apoptotic member of the Bcl-2 family, Bcl-X_L. The activation of NFκB, a well-studied transcription factor, has been linked to the prevention of apoptosis. It has been suggested that, in some cells, TNFRI induces NF-κB which then inhibits apoptosis by the transcriptional induction of the c-IAP1 and c-IAP2 caspase inhibitors. The induction of anti-apoptotic Bcl-2 family proteins, the induced degradation of pro-apoptotic Bcl-2 family proteins, and the expression of caspase inhibitors such as the IAP proteins represent some of the mechanisms by which apoptosis may be held at bay in cells that acquire a memory phenotype.

Ending T cell responses and Activation Induced Cell Death

By using artificially generated reagents such as labeled MHC class I-peptide tetramers, it has been possible to follow the fate of antigen specific CD8⁺T cells in infected mice and in human patients. It is now recognized that during an active immune response a very large number of the T cells in the host represent the clonal outgrowth of a few antigen specific CD4⁺ or CD8⁺ T cells. Examination of these clones has revealed that a large proportion of activated T cells are in the process of undergoing apoptosis. While large numbers of T cells do get activated by antigen most of these cells probably die because the amount of antigen is limiting and these cells no longer receive survival signals. Another major way in which activated T cells may be rendered quiescent is by the induction of inhibitory signaling via CTLA-4. Some cells which are restimulated by antigen also receive signals to die.

AICD is probably as important for the elimination of activated CD8⁺ cells as it is for the elimination of CD4⁺ cells. However it has been examined mainly in the context of CD4⁺ cells. Evidence from *lpr* and *gld* mice, from human lymphoproliferative syndromes involving Fas mutations, and from IL-2 and IL-2R α chain knockout mice has all come together to suggest the following scenario. When both Signal One and Signal Two combine to generate maximal T cell activation, transcriptional induction of both the IL-2 gene and of the IL-2R is achieved. This form of complete activation leads to signal transduction via the IL-2R and the maximal induction of both CD40L and FasL. During the course of these events the T cell makes cytokines, and may activate specific B cells and professional APCs via CD40L-CD40 interactions as well as by triggering specific cytokine receptors.

The majority of properly activated T cells commit suicide. They do so because the FasL induced on individual T cells triggers Fas receptors expressed by the same cell and thus induces death. Signaling via the IL-2 R is critical for the induction of AICD, probably because signals delivered via this cytokine receptor are required for the maximal induction of FasL. In addition IL-2 signaling may contribute to an inhibition of the cellular levels of FLIP. FLIP is a protein that is structurally similar to Caspase-8 but which lacks proteolytic activity. It can prevent the recruitment of Caspase-8 to FADD and thus protect a cell from Fas induced apoptosis. The reduction of FLIP levels by IL-2 may

be key in allowing the suicide process to go through successfully.

In CD8⁺T cells AICD might require signals to be delivered both by IL-2 as well as by TNF- α . Given that "proper" activation of a T cell leads to death, it is pertinent to ask how memory T cells are ever generated.

Making the choice between activation and memory: memory T cells

The existence of "true" memory lymphocytes has often been questioned and while their existence remains controversial, there is a growing acceptance of the view that such cells do exist. The persistence of antigen, either preserved by a low-grade viral infection or as part of long-lived immune complexes sequestered by follicular dendritic cells, could potentially contribute to the identification of recently restimulated cells as "memory" cells. The best evidence for the existence of "true" memory cells has come from studies on the transfer of CD8⁺ cells in the apparent absence of antigen which can result in the transfer of immune responsiveness for extended time periods. Although many of the central issues regarding memory remain controversial, there is some evidence to suggest that memory CD8⁺ cells can be maintained by being "tickled" via their TCRs even by non-specific, MHC class I-peptide complexes. Antigen, even if it is only required in a cross-reactive non-specific form, may well be required for the maintenance of what may well be "true" memory T cells. These cells may be best described as long-lived antigen specific T cells that emerge after activation by specific antigen but which do not require specific antigen for their extended *in vivo* survival.

The following general features characterize memory T lymphocytes (whether or not they pass the litmus test of antigen-independent survival):

1. Memory cells apparently survive for a long time *in vivo* in the apparent absence of specific antigen. They may survive as long as the lifespan of some small vertebrates.
2. Memory lymphocytes generally present with an activated phenotype, but can be distinguished from effector cells on the basis of size and function. They are smaller than effector cells. Effector CD4⁺ cells may secrete large amounts of cytokines whereas memory CD4⁺ cells may need to be triggered in order to do so. Effector CD8⁺ cells may be able to kill *ex vivo* targets directly while memory cells need to be triggered in order to be able to kill.

3. Memory cells do not require antigen for survival
4. Memory T cells can be activated by signals which may be below the threshold for the activation of naive T cells. This may in part be due to the high levels of adhesion molecules expressed on memory T cells.
5. Most memory cells probably live for a long time because they have been programmed by antigen exposure to express high levels of survival factors which include but may not be restricted to members of the Bcl-2 family.

Memory T cells may be categorized, into "central" memory cells which express CCR7 and traffic like naïve T cells to lymph nodes, and into "effector" memory cells which do not express CCR7 and return to tissue sites. The vast majority of effector T cells that are generated during an immune response are eliminated by AICD. A number of models have been proposed to explain how memory cells are generated. The most likely scenario is as follows: Following T cell activation a very large number of effector T cells are generated. As antigen is cleared, most of the vigorously activated T cells either die because they are no longer being stimulated or undergo AICD. A small number of these cells, perhaps those that were not activated as well because they arrived late on the scene, or those which were activated by distinct APCs in a slightly less vehement manner, fail to induce the suicide pathway and may go on to become memory cells. For CD8+ cells it has been established that effector cells go on to become memory cells. It is also possible that certain effectors are induced to "rest" and become memory cells, or that distinct activated cells give rise to effector cells and memory cells

The exact molecular pathways that are responsible for memory cell generation remain to be identified. It is very likely that anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL are induced by antigen receptor and costimulatory signals. Costimulation through CD28 may be required for the induction of Bcl-XL. The induction of cytosolic kinases such as Akt might contribute to the phosphorylation and inactivation of pro-apoptotic Bcl-2 family members such as Bad.

It is possible that much of the memory phenotype may be explained by two critical sets of biochemical changes:

1. Memory cells can be much more easily triggered through the TCR This might be because memory cells express higher levels of adhesion molecules than naive cells or because of alterations in intracellular signaling pathways.

This may explain their ability to continually receive survival signals when tickled even by "non-specific" MHC-peptide complexes. Genes downstream of TCR signaling may be in an "open chromatin" configuration in memory cells thus allowing low-intensity signals to trigger these cells, or to dramatically shorten the G1 period in cell cycle progression following a mitogenic stimulus.

2. Possibly as a result of being constantly "tickled", memory T cells express higher levels of anti-apoptotic proteins, or appropriately inactivate pro-apoptotic proteins.

Summary:

- On a molecular level, two major pathways for apoptosis are known. Both pathways include caspases, which are cytosolic proteins activated by proteolytic cleavage after specific aspartate residues. The first involves signaling at the surface with death receptors (Fas being a key example) that induces activation of caspase-8 via a multi-step process. Caspase-8 then activates caspase-3, which executes apoptosis via an unclear mechanism.
- The second molecular pathway may be induced by growth factor withdrawal, antigen receptor engagement, DNA damage etc., and involves the induction of small BH3-only proteins that activate Bak and Bax and thus cause proteins to leak out of mitochondria. Bak and Bax are opposed by anti-apoptotic proteins like Bcl-2. Released cytochrome c binds to APAF-1 and activates caspase-9 which then activates downstream caspases .
- Apoptosis in the immune system occurs via three possible overarching mechanisms: (1) Direct activation of death (i.e., by granzymes released by CTLs and NK cells or via Fas signaling). (2) Indirect activation due to gene induction (i.e., ionizing radiation, DNA damage). (3) Removal of survival signals (i.e., lack of growth factors or of MHC binding).
- CTLs and NK cells release granzyme B into the cytosol of target cells (presumably via perforin channels). Granzyme B is a serine protease that activates the caspase cascade of cell death.

- Growth factors can work to inhibit the apoptotic pathways. For example, some growth factors activate PI3 kinase, which activates Akt. The activated protein Akt inactivates procaspase-9, inhibits FasL synthesis, and inhibits Bad.
- In an immune response, activated T cells express both CD40L and FasL on the cell surface. For B cells making Ig's specific for antigen, BCR signaling as well as the CD40/CD40L interaction provides the stimulus for activation. Fas/FasL interactions are negated by BCR signaling. However, bystander B cells do not have BCR signaling and the activated T cell will induce death of these B cells via the Fas/FasL interaction (despite the CD40/CD40L interaction). In this way, only B cells specific for antigen will proliferate and become activated.
- Memory cells behave like immortalized cells, with no requirement for antigen signaling to survive. These cells can respond more rapidly to antigen. These characteristics of memory cells are potentially due to an altered chromatin state of cytokine genes, a higher expression of adhesion factors which helps to lower the threshold needed for signaling, and a higher expression level of anti-apoptotic factors such as Bcl-2.

Selected Reviews

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