Review

Cell Death Mechanisms Induced by Cytotoxic Lymphocytes

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One of the functions of the immune system is to recognize and destroy abnormal or infected cells to maintain homeostasis. This is accomplished by cytotoxic lymphocytes. Cytotoxicity is a highly organized multifactor process. Here, we reviewed the apoptosis pathways induced by the two main cytotoxic lymphocyte subsets, natural killer (NK) cells and $CD8^+$ T cells. In base to recent experimental evidence, we reviewed NK receptors involved in recognition of target-cell, as well as lytic molecules such as perforin, granzymes-A and -B, and granulysin. In addition, we reviewed the Fas-FasL intercellular linkage mediated pathway, and briefly the cross-linking of tumor necrosis factor (TNF) and TNF receptor pathway. We discussed three models of possible molecular interaction between lytic molecules from effector cytotoxic cells and target-cell membrane to induction of apoptosis. *Cellular & Molecular Immunology*. 2009;6(1):15-25.

Key Words: apoptosis, cytotoxic cell, granule-dependent exocytosis, FasL, TNF

Introduction

Cytotoxic function constitutes an important part of the cell-mediated immune system. Cytotoxicity is a highly organized multifactor process performed by different cells from the immune system. This process consists in inducing target cell death through cytotoxic effector cells. Natural killer (NK) cells and $CD8^+$ T cells are two of the main cell populations considered as cytotoxic cells, because their most important activity is to remove abnormal or infected cells to prevent the development of malignancies, and to eliminate intracellular pathogens (1-4).

NK cells are large, granular lymphocytes that mediate crucial functions of innate immunity mainly against viral infections (4). NK cells destroy target cells negatively or deficiently expressing classical and non-classical major histocompatibility complex class I (MHC-I) molecules, such as human leukocyte antigens HLA-A, HLA-B, HLA-C, HLA-E and HLA-G (5-8). Somatic cells express HLA-A, -B, -C, and -E, whereas HLA-G is found in the human placenta (9). NK cell cytolytic activity is also induced through the expression of cell stress-induced MHC class I-related chain A (MICA) and B (MICB) proteins (10). In contrast, $CD8^+$ T cells kill target cells by recognition of MHC-I restricted peptide antigens or CD1-restricted nonpeptide antigens (11, 12). For the recognition of antigens, $CD8^+$ T cells use an antigen-specific receptor, which is the outcome of genomic segment recombination during the development (13).

NK cell activity occurs in an antigen-nonspecific manner mediated by a wide range of inhibitory and activating surface receptors. These NK receptors have been grouped in: a) C-type lectin-like receptors (CD94 and NKG2 family); b) immunoglobulin-like transcripts (ILTs) or leucocyte immunoglobulin-like receptors (LILRs); and c) killer immunoglobulin-like receptors (KIRs).

Regarding the first group, CD94 molecule forms a hetero-dimeric complex with any member of the NKG2 family (NKG2-A, -B, -C, -D, -E, and -F). The activating or inhibitory function of the heterodimer complex depends on the cytoplasm region of NKG2 molecules. Accordingly, NKG2A and B have immunoreceptor tyrosine-based inhibition motif (ITIM) groups that act as inhibitors (14-16), whereas NKG2C, NKG2D, and NKG2E lack ITIMs, instead they transmit activating signals (17-19). NKG2C is associated with a 12 kDa DNA-activating protein (DAP-12), a factor containing an immunoreceptor tyrosine-based activating motif (ITAM) that provides cellular activating signals (17). Although NKG2D lacks ITAMs, it forms an activating receptor complex with a 10 kDa DAP (DAP-10), which contains a binding site for the recruitment of the phosphatidylinositol 3-kinase (PI3K) required for signal transduction (18, 20). NKG2E is similar to NKG2C in its cytoplasmic domain (19), which allows its association with DAP-12 (21). It seems that, NKG2F could regulate cell activation by competition for DAP12 with other receptors,

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such as NKG2C and NKG2E, although its exact function is not fully known (21).

NKG2A, B, C, E, or F receptors are expressed on NK cells, $CD8^+$ TCR αβ and γδ lymphocytes, $CD4^+$ T cell subsets, and NKT cells (1, 10, 22-25), all of which recognize HLA-E (17, 21, 26-28) complexed to an MHC class I leader sequence (29). In this fashion, they monitor the expression of MHC-I molecules on target cells. Likewise, NKG2D is also expressed on NK cells, γδ T cells, and $CD8^+$ αβ T cells, which recognize MICA and MICB ligands on stressed cells (30).

The second group of NK cell receptors corresponds to ILTs, also known as LILRs. The ILT cell surface molecules constitute a family of activating and inhibitory receptors expressed on myeloid and lymphoid cells. ILT1 (LIR7) is a receptor expressed on myeloid and lymphoid cells, but that lacks ITIMs (31). It has been reported that ILT1/LIR7 activates human eosinophils and basophils (32, 33). ILT7 is a similar activating receptor that has been proposed as a marker for plasmacytoid dendritic cells (34, 35). ILT1 and ILT7 are associated with the FcεRI-γ adaptor molecule in their cytoplasmic tails (31, 34). Regarding other members of this family, ILT2 (LIR1), ILT3, ILT4 (LIR2), and ILT5 are all polymorphic inhibitory receptors containing ITIMs in their cytoplasmic tails (36). ILT2 is expressed on NK cells, dendritic cells, and all T cells (37), whereas ILT4 is found mainly in monocytes (38). ILT2 and ILT4 bind preferentially to the nonclassic MHC molecule HLA-G, with a higher affinity than to classic MHC-I, suggesting that these molecules can regulate the immune response in the maternal-fetal interface (38-40). ILT3 is a receptor expressed on dendritic cells, monocytes, and endothelial cells (41). It has been reported that ILT3 expression on endothelial cells is induced by interleukin (IL)-10 and interferon (IFN)-α (42, 43). ILT6 is a receptor with scarce polymorphism (44) that activates T cell proliferation when produced by macrophages in a soluble form (45). Other receptors have also been identified, including ILT8, ILT9, and ILT10, but their functions are still unknown (46, 47).

Concerning KIRs, these molecules possess a characteristic structure of immunoglobulin domains in the extracellular region. They have also a cytoplasmic tail that determines their biological function (48). Long cytoplasmic domains contain ITIM groups that cause inhibitory effects, while short domains are activators and contain a lysine residue comparable to those from the NKG2 activator group to which DAP is linked. KIR molecules are denominated either short (S) or long (L), for example KIR3DL, based on the extracellular domain number and the length of the cytoplasm domain. Classic and non-classic MHC-I molecules are KIR ligands, such as HLA-G and HLA-Cw3, which are recognized by KIR2DL4 and KIR2DL2, respectively (6, 40).

In spite that $CD8^+$ T cells and NK cells differ in the target cell-recognition and activation mode, their effector functions are carried out in the same way (Figure 1). A final consequence from cytotoxic activity is target cell death. However, the exact nature of how cell death occurs is very important, since this determines the amount of damage inflicted upon neighboring cells. Cell death can be caused by either necrosis or apoptosis.

Necrosis: In this process, cells undergo irreversible morphological changes that take place in a fast and disorderly manner. A large number of intracellular components are released into the intercellular space due to cell burst. Necrosis causes damage to the surrounding tissue because it promotes an inflammatory process (49).

Apoptosis: Cells also undergo morphological changes, but these take place in a programmed and controlled fashion. Initially, there is a limited chemical alteration on the apoptotic cell membrane, followed by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum and, finally, a packaging of intracellular contents into cell bladders denominated "apoptotic bodies". These apoptotic bodies are then phagocyted by surrounding phagocytes, thus preventing the release of the cytoplasmic content to the outside. Hence, the development of an inflammatory response and damage to the neighboring cells are avoided (49, 50).

Apoptosis can be induced by three pathways: a) granule-dependent exocytosis pathway, b) Fas-FasL intercellular linkage-mediated pathway, and c) cross-linking of TNF and TNFR type I.

Granule-dependent exocytosis pathway

This pathway is established through intracellular signaling after target cell recognition by a cytotoxic lymphocyte (NK or cytotoxic T cell). In exocytosis or degranulation, there is microtubules mobilization that leads the preformed granules or lysosomes of the cytotoxic cell towards the point of contact with the target cell, releasing stored lytic molecules (51, 52). Degranulation can be detected by exposure of the lysosomal-membrane-associated glycoproteins, CD107a, CD107b, and CD63, on the lymphocyte surface (53). These molecules are found in the granule-membrane inner surface and are exposed onto the lymphocyte surface through degranulation (53-55). It has been suggested that lysosomal-membrane-associated glycoproteins and the soluble protein cathepsin-B play an important role in avoiding lymphocyte self-destruction. The lytic granule contains a proteoglycan matrix that maintains protease enzymes in an inactive stage (56). The lytic granules mature through an hMunc13-4-dependent maturation process that is required to efficiently release lytic molecules on the target cell (57). The lytic molecules stored in granules that induce apoptosis are perforin, granzymes (Grzs), and granulysin.

Perforin

The importance of this molecule has been evaluated in animal models or human disease observations. Mice with perforin deficiency develop spontaneous lymphoma (58) and, in patients with familial hematophagocytic lymphohistiocytosis, a mutation in the perforin gene causes evident diminution of their immune response to infections by intracellular pathogens (59).

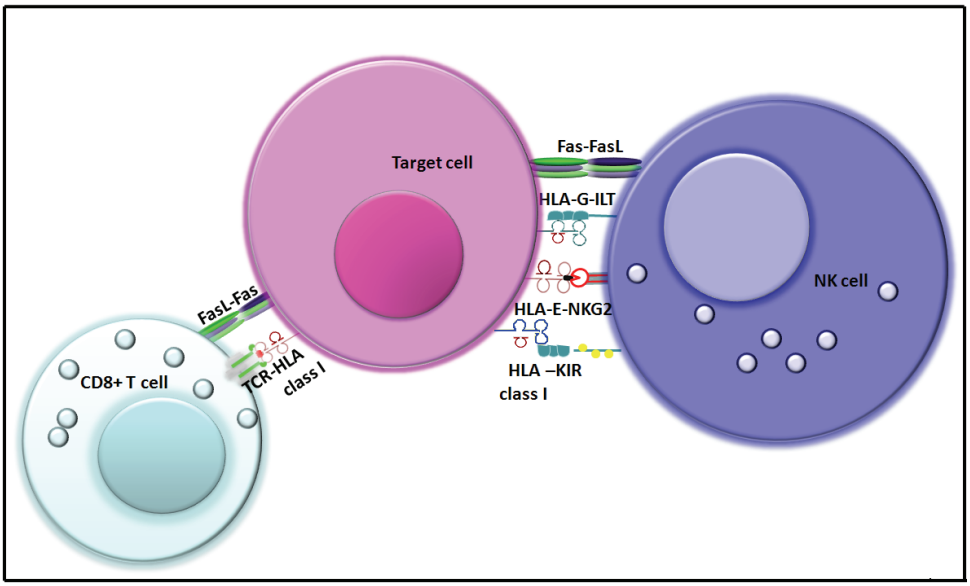

Figure 1. CD8$^+$ T cells and NK cells recognize the target cell through different receptors. Although both cytotoxic cells act upon target cells inducing apoptosis, the mechanism of lymphocytes mediated-cell recognition is different. NK cells kill target cells in an antigen-nonspecific manner regulated by a wide range of inhibitory and activating surface receptors. In contrast, CD8$^+$ T cells destroy target cells by recognition of HLA class I restricted peptide antigen. Fas molecule is expressed on target cells, whereas FasL molecule is expressed on cytotoxic cells. TCR, T cell receptor; ILT, immunoglobulin-like transcripts; NKG2, NK cell group 2 transmembrane receptors; KIR, killer immunoglobulin-like receptor.

Despite to be the first protein isolated from lytic granules, perforin has caused controversies because its structure, action mechanism, and synergism with other molecules are unclear (60). Structurally, perforin has an N-terminal domain with lytic ability, and then a region of 150 amino acid residues whose function remains unknown (61). In the middle of perforin there is an α-helix amphipathic domain that regulates its transmembranal insertion and confers the stability required to form pores upon the target cell membrane (62). Finally, it has a C-terminal domain, this region is able to make catenary's interactions of calcium-dependent membrane binding (63). The second domain of the latter region is essential for the binding between cell membrane and perforin; at the end of the 20 amino acid residues, there is a signal sequence for N-glycosydic linkage and a breaking site considered important for perforin activation (64).

Perforin is found in a soluble monomer shape within granules and, after the cytotoxic-cell/target-cell junction, perforin is released by exocytosis (54). Once it is anchored, perforin begins the polymerization in the presence of Ca^{2+} to form cylindrical pores with an internal diameter of 5 to 20 nm (63, 65, 66). The perforin pores can serve as passive conductos of granzymes and granulysin through the target cell membrane and could also allow an ionic exchange, which causes an osmotic unbalance and in consequence, the cell death (Figure 2A) (65). However, in spite of this being the most accepted hypothesis, there is little experimental evidence to support it. The controversy lies in the mechanism and function of perforin. Motyka et al. have shown that in the absence of perforin, granzyme (Grz)-B is introduced into the target cell because granzyme forms a complex with the mannose-6-phosphate receptor independently from cations. Subsequently, the complex is internalized by endocytosis. Once inside the cell, the granzymes and granulysin are released favoring interaction with their substrate to induce apoptosis (Figure 2B) (67). However, Trapani et al. suggest that the mannose receptor is not nessesary for the entry of Grz-B into the target cell (68). Keefe D et al. showed evidence that Grzs adhere to the cell surface by electrostatic linkage, whereas the perforin pore induces Ca^{2+} flow from the extracellular towards the intracellular environment (69). Ca^{2+} entrance causes activation in the target cell, which attempts to amend the pore in the cell membrane to avoid necrosis (69). In consequence, the Grzs are internalized together with perforin and are released in the cytoplasm of the target cell (Figure 2C). However, the presence of perforin is required to induce apoptosis (70). The evidence indicates that perhaps perforin is not essential for the entrance of proteases into the target cell, but it is required for cytolysis. In different proposed models, the function of perforin is not elucidated, and has been limited to cell surface, or to endosome membrane, or both. The query remains about how granzymes are released from the endosome (whether they are internalized by endocytosis) because their dimensions and molecular structure would prevent their passing through the perforin pore. It could be speculated at least a chemical or physical factor (pH, other proteins, etc.) originated from the intracellular trade, might be altering the structure and/or the size of either the Grzs or the pore (Figure 2).

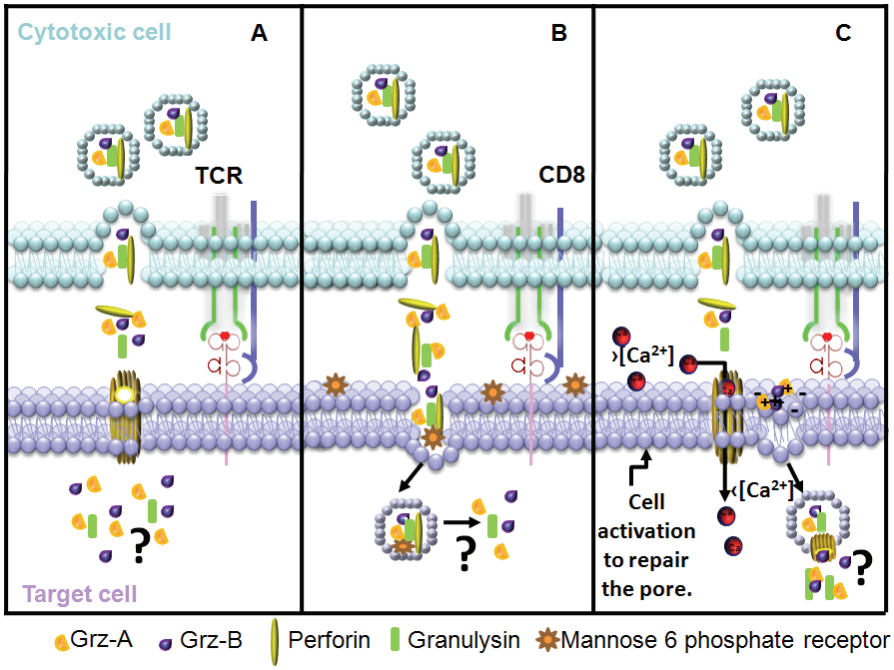

Figure 2. Three proposed models for internalization of lytic molecules. (A) Perforin polymerizes on the target cell membrane forming a pore through which granzymes (Grz)-A, -B, and granulysin enter to induce apoptosis. (B) The target cell membrane expresses mannose-6-phosphate receptor that forms a complex with Grz-B, which is then internalized together with Grz-A and granulysin inside vesicles and released by pore-forming perforin into the cytoplasm, where these molecules exert their function. (C) The pore formed by perforin allows the entry of extracellular Ca^{2+} activating the target cell, which attempts to repair the damage and endocytoses the membrane region together with lytic molecules adhered by electrostatic linkages. TCR, T cell receptor; red sphere, Ca^{2+} ions; > [Ca^{2+}], high concentration of calcium ions; < [Ca^{2+}], low concentration of calcium ions.

Concentration of the perforin released at the contact site between the cytotoxic effector cell and the target cell is still unknown. For functional studies on the role of perforin, dose-response assays are needed, which, in consequence could help to determine its optimal concentration for future experiments. At the sublytic concentration of perforin, a 10% necrotic cell death is observed, and the range varies from 50 to 500 ng/ml. At a lower concentration, the granzymes are not released, whereas, at a higher concentration, perforin causes necrosis on most cells. Perforin is an unstable molecule; therefore, the amounts vary according to the cytotoxic cell population (69, 71).

Granzymes

Granzymes are soluble proteins of a globular structure, belonging to the serine-proteases family. Grz-A and Grz-B are the most abundant within lytic granules (72). Grazymes are released as a multi-molecular complex, inducing apoptosis by caspases-independent or -dependent pathways (71). Caspases are found in cell cytoplasm as inactive precursor molecules that need to be hydrolyzed to begin their activity. According to their function, Grzs are grouped as follows: a) inflammatory, b) initiator (of stress signals), and c) effector (of apoptosis) (71, 73).

Grz-A induces caspase-independent apoptosis, activating a slow process of cell death. It cleaves single-stranded DNA, and hydrolyzes proteins containing basic amino acids such as arginine or lysine (74, 75). Grz-A activates an endoplasmic reticulum associated complex (the SET complex), which is conformed by two tumor-suppressor proteins, phospho-protein 32 (pp32) and nonmetastatic protein 23 homologue 1 (NM23-H1), and three Grz-A substrates: oncoprotein SET, high mobility group 2 (HMG-2) protein, and apurinic endonuclease 1 (Ape1) (74, 76, 77). A characteristic of apoptosis is the increase of reactive oxygen species (ROS) and decrease of the mitochondrial membrane potential, a process that seems to play a pivotal role in the SET translocation into cell nucleus *via* mechanism that is not fully understood (Figure 3) (74, 75, 78). Once inside the cell nucleus, Grz-A cleaves SET (specific inhibitor for NM23-H1), and the cleavage of SET releases NM23-H1, which degrades chromosomal DNA (Figure 3) (74, 76). It is also postulated that Grz-A cleaves to histone 1, modifying the nucleosomal center, so chromatin is relaxed and DNA is fragmented by endonucleases (79). Extracellular activity of Grz-A has been also reported by either fragmenting the IL-1β pro-peptide at the Asp116 site to give rise to the active form of IL-1β or activating fibroblasts to secrete cytokines, such as IL-6, IL-8 and IFN-γ (74, 76, 80, 81).

Grz-B cleaves protein substrates in the carboxyl side on acidic amino acids, especially aspartic acid; some Grz-B substrates appear in pre- or post-mitochondrial phase (71).

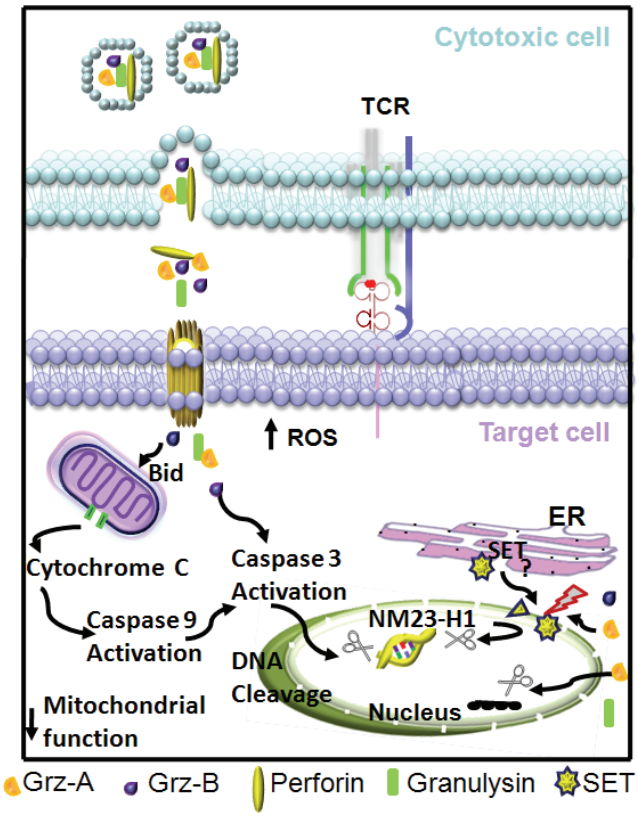

Figure 3. Mechanisms by which lytic molecules act upon target cell. Once inside the target cell, Grz-A activates an endoplasmic reticulum (ER) associated complex, which is conformed by phosphoprotein 32 (pp32), nonmetastatic protein 23 homologue 1 (NM23-H1), oncoprotein SET, high mobility group 2 (HMG-2) protein, and apurinic endonuclease 1 (Ape1). The increase of reactive oxygen species (ROS) and less of the mitochondrial membrane potential induce the translocation of the SET/NM23-H1 complex into cell nucleus by an unclear mechanism. Then, Grz-A cleaves SET, which activates NM23-H1, an endonuclease that fragments chromosomal DNA. In addition, Grz-B cleaves Bid or activates caspase 3 directly, which degrades DNA. Truncated-Bid, in turn, causes permeability of the mitochondrial outer membrane and cytochrome-C release with a diminution of mitochondrial function. Cytochrome-C induces activation of caspase 9 that enhances the apoptosis process by downstream activation of caspase 3. TCR, T cell receptor.

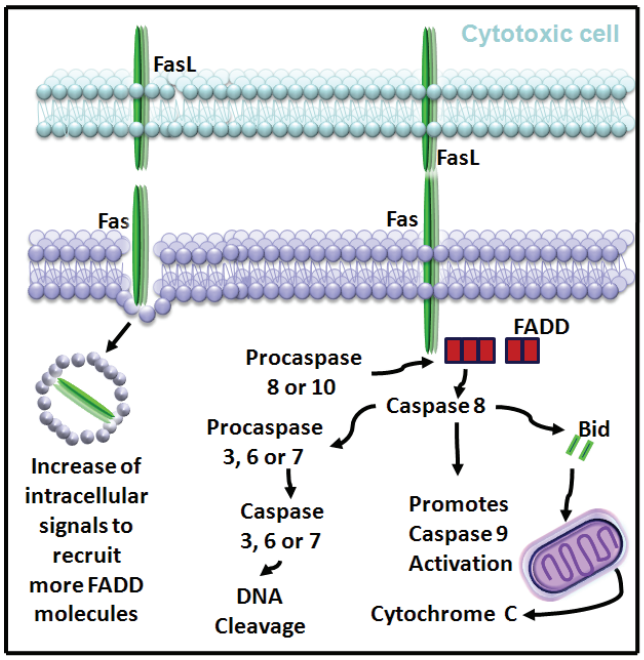

Figure 4. Apoptosis mediated by Fas-FasL pathway. The interaction of the FasL trimer with Fas induces the trimerization of Fas-associated death domain (FADD) molecules that recruit procaspase 8 or 10. Then, procaspase 8 or 10 is activated. Increase of intracellular signaling enhances the recruitment of FADD molecules. Caspase 8 activates procaspases 3, 6 or 7, and activates Bid promoting cytochrome-C release. Caspase 8 also cleaves procaspase 9, which also becomes activated. Finally, activated caspases 3, 6, and 7, degrade chromosomal DNA leading to target cell death.

Grz-B can induce apoptosis rapidly through two pathways. In the first, it activates caspase 3 directly, which promotes the fragmentation of DNA, or of nuclear membrane crucial components or of the cytoskeleton (82, 83). In the second pathway, Grz-B promotes permeability of the mitochondrial outer membrane and cleaves Bid, a molecule from the Bcl-2 family (82). In turn, Bid induces cytochrome-C release from mitochondria and other apoptogenic intermembrane molecules, such as HtrA2/Omi, endoG, and AIF into the cytoplasm (84). Cytochrome-C is important to trigger the formation of apoptosomes and activation of caspase 9, which enhances caspase 3 activation, decreasing mitochondrial function and, in consequence, causing cell death (83) (Figure 3). It has been reported that caspase 8 is activated under physiologic conditions in order to accelerate the target cell destruction (82). Some authors propose that Grz-B preferentially triggers apoptosis due to an alteration of the mitochondrial membrane rather than by the direct action of caspases (71, 73). However, it continues to be a controversial topic.

Although it has been reported that the activity from both granzymes, Grz-A and Grz-B, is dependent on cathepsin-C, Sutton et al. observed that in cathepsin-C-knockout mice, apoptosis is induced by Grz-B, pointing out that only Grz-A is cathepsin-dependent, suggesting that wild-type cytotoxic cells secrete more Grz-B for DNA fragmentation in a caspase-dependent manner (85).

Lytic granule contains also orphan granzymes, but their function is less been defined. However, these molecules are essential for cytolysis of the target cell in knockout mice. In humans, Grz-K is a trypsin-like protease that induces apoptosis by Bid-dependent mitochondrial outer membrane damage (86). Grz-H is a chymotrypsin-like protease (chymase) that seems to have synergistic functions with Graz-B (87). Grz-M is a serine protease highly expressed in NK cells, and it induces cell death without DNA fragmentation (88). Grz-M cleaves the actin-plasma membrane

linker, ezrin, and the microtubule component α-tubulin, disorganizing the microtubule network (89). In mice, there are other granzymes such as C, D, E, F, G, and K; and rats have granzymes C, I, K and M, which show distinct structural and functional characteristics (90).

Granulysin

As a cytolytic molecule and member of the saposin-like proteins family, granulysin is a small cationic protein encoded in the human chromosome 2 (there is no homologous molecule in the mouse). Granulysin is stored in granules from NK cells, cytotoxic T cells, helper T cells, and NKT cells; its active form weighs 9 kDa and it is expressed from 3 to 5 days after cell activation (91). Because granulysin has a structural similarity to the saposins. It has been suggested that its lytic activity occurs when its interactions, mainly with negative charges from target-cell mitochondrial membrane lipids, induce cell membrane damage (91). This mechanism induces release of cytochrome-C and decreases of mitochondrial function, which is related with the perforin-pore (92). Recently, Walch et al. showed that the granulysin-dependent cell lysis augments in the presence of perforin (93). Another mechanism by which this molecule induces cell death is through caspase-3 activation (92). The function of granulysin is not only limited to cytolysis but is also a leukocyte chemoattractant or activator at nano-molar concentrations in an inflammatory environment (94). In just micromolar concentrations, granulysin causes cellular lysis, thus this molecule plays an important role in the field of rejecting allografts (95). It has also been reported that granulysin level in blood serum is an important marker of immunological status in gastric carcinoma patients (96).

Fas-FasL intercellular linkage-mediated pathway

This apoptosis pathway is important in the control of constantly stimulated T cells, and in promoting tolerance to self-antigens, aside from being a homeostatic mechanism of the cytotoxic T cell activity. In defects of this pathway, mice develop lymphoproliferative disorders, and humans develop the autoimmune lymphoproliferative syndrome, of which there are various types (Ia, Ib, II, III, and IV) depending on the site at which the mutation is found. All of these processes are controlled by one punctual mutation (97). Other pathologic disorders reflect the importance of molecules involved in this pathway; for instance, when mice *lpr* and *gdl* genes are altered (human Fas and FasL homologous), they develop proliferative disease of the renal tubule (98). Likewise, there is over-expression of Fas in infected $CD4^+$ T cells from patients with HIV (99).

Effector T cells and NK cells express FasL (CD178), whereas target cells express Fas (CD95 or Apo-1), thus these cells are susceptible to apoptosis mediated by this pathway (100, 101). Fas molecule is a cell surface protein that weighs 45 kDa. It was identified by an apoptosis-inducing antibody on human cell lines (102). Fas belongs to the tumor necrosis factor receptor (TNFR)-I type family. It has one extracellular domain rich in cysteins that binds FasL, and another cytoplasm domain involved in death signals (103). FasL is an inductive molecule expressed on T cells, and weighs 40 kDa. It is homologous to the cytokine tumor necrosis factor (TNF), and is a member of the TNFR-II type family (102). FasL is constitutively expressed on cells of immune privileged organs, such as brain, anterior chamber of eyes, and testes. In consequence, FasL protects these privileged sites from the action of immune system cells, as an additional regulatory mechanism of self-tolerance (104). The FasL expression depends on the transcription factors level. The positive regulators are NFAT, Egr2/Egr3, NFκB, AP-1, c-myc SP1, and B1/Cdk1, whereas the negative regulators are c-Fos and CIITA (105-107). Some regulatory factors function by binding directly to FasL DNA, while others indirectly regulate transcription factors (107).

FasL can be expressed in three ways: the first as highly arranged trimers anchored on the cell surface membrane, it is a primary mediator of apoptosis. In the second, FasL is anchored to intracellular membrane microvesicles, where it is stored until expressed on the cell surface in response to physiological stimuli. The last corresponds to a soluble FasL, which is generated by degradation of the membranous shape (during the first minutes of expression) due to the activity of a metalloprotease matrix whose function is to catalyze the degradation of extracellular matrix proteins (108-111). The soluble FasL molecule has either pro-apoptotic or anti-apoptotic properties since soluble FasL is an inefficient homotrimer binding to Fas. When these molecules interact, the outcome is null signaling with no apoptosis. On the contrary, soluble FasL can induce apoptosis after its association or aggregation with extracellular matrix proteins. In addition, apoptosis is induced when soluble FasL forms tetramers or highly arranged structures (111). Fas/FasL pathway plays an important role in graft rejection (112), where soluble FasL can be chemotactic to neutrophils, during the acute rejection of a graft transplant (113).

Binding of Fas with FasL causes trimerization and recruitment of Fas-associated death domain (FADD) proteins through homotypic death domain interactions. In turn, trimerized FADD recruits either procaspase 8 or 10, which undergo a process of autoproteolysis to become an activated caspase (114). Assembly of these components results in the formation of a death-inducing signaling complex (DISC), which is pivotal in the receptors-dependent apoptosis (114). Caspase 8 interacts with procaspases 3, 6, or 7 and, through a process of transproteolysis, they become activated caspases. Finally, these effector caspases cleave DNA (Figure 4). Caspase 8 can also hydrolyze Bid, which causes damage to the mitochondrial outer membrane and trigger cytochrome-C release (115, 116) (Figure 4).

Besides FasL, another member of the TNFR family is the TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo-2L (117). TRAIL has two receptors, TRAIL-R1 (death receptor 4, DR4) and TRAIL-R2 (DR5), which belong to the TNFR family too (118, 119). Once linked to TRAIL,

these receptors engage FADD proteins in their cytoplasmic portion. Then FADD proteins recruit procaspase 8 that is activated within the DISC (120, 121). Caspase 8 is able to trigger the apoptosis process either through interaction with procaspases 3, 6, 7 or Bid cleavage, as mentioned above.

It has been reported that induction of apoptosis by death receptors occurs in two modes, dependent on the cell type. Cells that die due to apoptosis accompanied by large amounts of active caspase-8 originated at the DISC are denominated type I, whereas cells wherein receptor-mediated death relies mostly on the release of pro-apoptotic factors from the mitochondria are denominated type II (122). Type I cells rapidly internalize Fas into an endosomal compartment in a clathrin-actin dependent manner (123), which is a requirement to assemble of DISC components. Thus, type I cells require Fas internalization to enhance signaling events toward the apoptosis process (124). In contrast, FasL-stimulated type II cells require amplifying the apoptosis signal through a contribution from the mitochondria (123). Recently, it has been shown that FasL-induced endocytic vesicles reach the mitochondrial compartment leading to type II cell demise (125).

Regarding TRAIL receptors, the same biological phenomenon appears to occur. Kohlhaas et al. reported that the TRAIL receptor internalization is not necessary for DISC formation and apoptosis induction (126). However, the TRAIL-induced apoptosis entails a loss of mitochondrial membrane potential (127) perhaps by caspase 8 activity. Caspase 8 can induce a loss of mitochondrial membrane potential, which promotes releasing of cytochrome-C (83, 122). The cytochrome-C is important to trigger apoptosis process in the target cell by downstream activation of caspase 3 (83, 128) (Figure 4).

One evident difference between the Fas-FasL intercellular linkage-mediated pathway and the granule-dependent exocytosis pathway is the persistent induction of apoptosis by FasL in cytotoxic T cells. The elimination of FasL from the cell surface requires 2 to 3 hours, this period permits to continue exerting cytotoxicity even in the absence of stimulus *via* TCR (129). Thus, Fas-expressing neighbor cells could be eliminated, though they did not express the specific antigen recognized by T cytotoxic cell. This explains the promiscuity of this pathway as compared to the granule-dependent pathway (130). Recently, Cunningham et al. showed that PI-9 (proteinase inhibitor 9) expression, an inhibitor of the human Grz-B, blocks cytotoxicity exerted by both the Fas-FasL pathway and the granule pathway in cell lines (131). This suggests that granzymes play an important role in the death receptor pathway or that PI-9 acts in an additional manner on caspases.

Cross-linking of TNF and TNFR type I dependent pathway

TNF is a cytokine produced by activated cells that induces cell apoptosis, inflammatory processes, cell activation, and differentiation (132, 133). The TNF molecule can induce receptor oligomerization increasing the ligand binding affinity (134). The receptors of TNF (TNFR) can be grouped into three classes: 1) having cytoplasmic death domains, 2) linked to adaptor molecules denominated TNF receptor associated factors (TRAFs), and 3) soluble receptors (135, 136).

In TNF-induced apoptosis by contribution of FADD molecules, initially after interaction with TNF, the TNFR undergoes multimerization to form the DISC signaling downstream through the caspase activation cascade and mitochondrial changes (137). Moreover, the TNF-TNFR complex also leads to the recruitment of TRAF molecules that signal downstream, activating the transcription factors NF-κB and JNK (138, 139). Chandel et al. suggest that TRAFs play an important role in regulating the increase of intracellular reactive oxygen species (ROS), and that TRAFs regulate the cellular redox status (140).

TNF-TNFR complex has also been shown to enhance NADPH oxidase activity promoting a burst of oxidative stress and leading to necrotic cell death (141, 142).

Summary

Cytotoxicity is an activity performed by specialized cells such as NK cells and $CD8^+$ T cells. NK cells show diverse receptors for the recognition of altered-cells; these receptors activate or inhibit NK cell-cytotoxicity depending on signalization by ITAM or ITIM groups in the cytoplasmic portion, respectively.

NK cells and $CD8^+$ T cells, despite of acting upon target cells in the same way, inducing apoptosis, the mechanisms of lymphocytes mediated-cell recognition are different. NK cells destroy target-cells deficiently expressing molecules involved in antigen presentation, such as classic and non-classic MHC. This NK cell activity occurs independently of the presented specific antigen. In contrast, cytotoxic T cells kill target-cells processing and presenting specific antigen through MHC class I or CD1 molecules.

Apoptosis can be induced by three pathways: a) granule-dependent exocytosis pathway, b) Fas-FasL intercellular linkage-mediated pathway, and c) cross-linking of TNF and TNFR type I. In the first pathway, lytic molecules such as perforin, granzymes, and granulysin participate. However, just how these molecules access the target-cell has not been clearly elucidated so far. In the second and third pathways, Fas or TNFR receptors trimerization is required to lead intracellular signalization towards the apoptosis process.

Elucidation of the molecular mechanisms of apoptosis will allow gaining precise knowledge on the participating phenomena. On the other side, their elucidation will shed light on the biological problems involved in infectious and lymphoproliferative processes.

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