Death Pathways in T Cell Homeostasis and Their Role in Autoimmune Diabetes

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Abstract

T cell apoptosis is a process necessary for central and peripheral tolerance. It ensures the proper removal of autoreactive T cells during thymic development as well as T cell homeostasis and the downregulation of immune responses against antigens in the periphery. Thus it is essential for the prevention of autoimmunity. Apoptotic pathways can be triggered by intrinsic (mitochondria-based) and extrinsic (receptor-based) stimuli. Both pathways involve a cascade of proteolytic enzymes called caspases whose activation commits the cell to death. In the periphery, autoreactive lymphocytes can be silenced by developmental arrest (anergy), or deleted by programmed cell death (apoptosis) through receptor-based activation-induced cell death (AICD). Central tolerance seems to rely more heavily on the mitochondria-based, T cell receptor (TCR)-stimulated apoptotic pathway, since thymocytes lacking the pro-apoptotic Bcl-2 family member Bim are resistant to TCR-induced apoptosis. Furthermore, defects in the intrinsic pathway of apoptosis may impair clonal deletion of autoreactive T cells. Several animal models exist in which impaired apoptosis results in autoimmunity. Here, we discuss data that suggest defects in T cell apoptosis in type 1 diabetes mellitus.

Keywords: type 1 diabetes · T cell · apoptosis · programmed cell death · mitochondria · death receptor · Fas

Introduction

T cell homeostasis and fully functional T cell compartments are critical to achieve an effective defense against foreign antigens and to avoid overreactivity against self-antigens [1]. This is achieved through processes mediating proliferation and elimination of lymphocytes. Signaling through antigen receptors promotes survival, proliferative expansion and differentiation of useful and deletion of useless and dangerous T cells [2].

Apoptosis is necessary to control the development of T cells and to maintain the T cell repertoire in a balanced state (homeostasis). It thus acts in two ways. Firstly, T cell development and maturation in the thymus require education and selection mechanisms. T cells that can recognize and remain unresponsive against self major histocompatibility complex (MHC) and self-antigens are positively and those that cannot recognize or that are even activated by interactions with self-MHC and self-antigens are negatively selected and die by apoptosis [3]. Thymic apoptosis programs are thus necessary for the selection of functional, correctly equipped and sensitive T cells, while at the same time, to avoid the escape of degenerated (e.g. insensitive or equipped with wrong markers), incompletely developed (e.g. with missing pre-T cell receptor (TCR) α or β) or autoreactive T cells [4, 5].

Secondly, during immune responses, activated and pathogen-specific peripheral T cells, which matured in

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the thymus and were exported to the periphery, undergo extensive expansion and proliferation and develop effector functions, such as cytotoxicity or cytokine production [6]. When infectious or foreign tissue cells are eliminated by a T cell-mediated immune response, most of the activated T cells are removed by a process called programmed cell death (PCD), also called apoptosis, in order to prevent damage of own tissue by these effector cells and the released cytotoxic molecules [7, 8]. The shift towards a pro-apoptotic milieu at the end of an immune response is achieved by limiting the provision of growth factors such as IL-2, and a decrease in the expression of anti-apoptotic Bcl-2 members, a mitochondria-dependent protein family containing members that can be pro- or anti-apoptotic [9, 10]. The remaining T cells survive. Upon withdrawal of stimulation, they assume a memory phenotype and live on as trained quiescent lymphocytes in peripheral lymph nodes [11]. For the survival of mature resting T cells and those with memory functions, costimulation by CD28 ligation and IL-2 expression, which is increased by costimulation, together with anti-apoptotic Bcl-2 expression is necessary [12-14]. In combination with other mechanisms, including developmental arrest or induction of unresponsiveness (anergy), PCD of autoreactive lymphocytes safeguards immunological tolerance to self-antigens and assists in the development and maintenance of an effective and tolerant immune system [2].

In autoimmune diabetes, activated $CD4^+$ and $CD8^+$ T cells proceed to kill self-tissue after an early event of pancreatic inflammation by peripheral blood monocytes and macrophages [15]. Even though it is not clear whether autoimmunity is initiated as a consequence of an environmental trigger such as a virus infection, molecular mimicry, abnormal TCR or something different, genetic predisposition is suspected to play a decisive role in the development of type 1 diabetes (T1DM) [16, 17]. This phenomenon points to an intrinsic failure in antigen recognition, processing or T cell selection and elimination. In any case, islet antigens, such as glutamic acid decarboxylase (GAD), insulin, proinsulin and tyrosine phosphatase (IA-2), are recognized as foreign and are antagonized by a T-cell-mediated immune response [18, 19]. After activation of islet-specific $CD4^+$ and $CD8^+$ T cell populations by cell-to-cell contact with APC and their subsequent proliferation, the destruction of pancreatic β-cells occurs. The process involves a large number of self-reactive T cells and the question arises why these cells escape thymic or peripheral tolerance mechanisms. It seems that in diabetic patients either thymic selection or PCD after an immune response is defective. Defects in thymic selection could be observed in NOD mice [20] and there is also evidence for defects in peripheral activation-induced cell death (AICD) after immune response [21, 22]. Beside PCD, other mechanisms mediating tolerance are also suspected to bear defects in T1DM, e.g. disturbances in the activity of regulatory T cells of the $CD4^+CD25^+$ phenotype, which control potentially self-reactive T cells [23]. However, this article focuses on the mechanisms that control PCD and discusses possible failures in this process that could play a role in T1DM development as well as potential strategies to overcome them.

Regulation of T cell homeostasis by apoptosis and the role of death signals

Naïve and memory T cells exist as silent lymphocytes in the periphery where they are maintained by the provision of stimulation, growth factors such as IL-2 and a high internal level of anti-apoptotic Bcl-2 [14, 24, 25]. Following an immune response most of the activated T cells need to be deleted by apoptosis, which is induced by a switch from an apoptosis-resistant to an apoptosis-sensitive state. This can be mediated by cytokines, death receptors and pro-apoptotic proteins [26].

The process of PCD can be initiated by two different but interlinked pathways: an intrinsic, mitochondria-dependent and an extrinsic, receptor-induced pathway. Despite different modes of initiation, an activation of a cascade of proteolytic enzymes, termed cysteine proteases or simply caspases, occurs. Once initiator caspases (8, 9 and 10) are activated, the cell is committed to death. Both pathways play critical roles in terminating immune responses [27].

Death receptor-induced apoptosis

The extrinsic pathway of apoptosis, also called activation-induced cell death (AICD), is initiated by ligation of membrane-bound trimeric death receptors such as CD95/Fas, tumor necrosis factor receptor (TNFR) and death receptors (DR) 4 and 5, which are expressed upon TCR stimulation [28, 29]. Death receptor ligation either by CD95L (FasL), TNF or TNFR-related apoptosis-inducing ligand (TRAIL, also called Apo2L) leads to caspase 8 activation, procaspase 3 cleavage and subsequently to cell death [26, 29]. Caspases exist within a cell as zymogens, i.e. inactive precursors called procaspases. Activation of a procaspase can activate effector caspases (3 and 7) downstream in the activation cascade. Full activation of these effector caspases eventu-

ally leads to nuclear condensation, DNA fragmentation and cell death (Figure 1) [30, 31].

Death receptors contain a so-called death domain (DD) in their cytoplasmic tail and, depending on the receptor the DDs are associated with; they have been named Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD). DDs are able to recruit initiator caspases 8 and 10 upon binding through their death effector domain (DED) to an analogous domain repeated in tandem within the zymogen form of the caspases. Sensitivity to FasL- as well as TNF-mediated cell death is thus controlled by the so-called death-inducing signaling complex (DISC), which contains the adaptor protein FADD and the initiator caspases 8 and 10. TNF-mediated apoptosis includes the TRADD in addition to the DISC (Figure 1) [26, 30].

AICD is important to downscale immune responses after activation and clonal expansion of antigen-specific T cells [32]. T cells are resistant to receptor-mediated apoptosis until they are fully activated. This resistance is due to intracellular proteins that inhibit or block death signaling within the cell. The caspase 8-homologous FLICE (Fas-associated death-domain-like interleukin 1β-converting enzyme) can be blocked by cellular FLICE inhibitory protein (c-FLIP) [33-35]. If death receptor-induced stimulation is blocked or not sufficient to execute full effector caspase activity, caspase 8 can mediate the cleavage of the pro-apoptotic Bcl-2 member Bid towards truncated Bid (tBid) and thereby initiate the intrinsic pathway of apoptosis [36, 37].

Mitochondria-dependent apoptosis

The mitochondria-dependent apoptotic pathway is mainly controlled by the balance between pro- and anti-apoptotic members of the Bcl-2 protein family [38]. A critical event in the apoptotic process is the change of the mitochondrial membrane potential provoked by the Bcl-2 family of proteins that either protect against or induce membrane depolarization and permeabilization [39]. Depolarization, once induced, is a reversible but strong initiator of the intrinsic apoptotic program. Subsequently, cytochrome C is released by the mitochondria and causes the adaptor protein apoptotic protease-activating factor 1 (Apaf-1) to activate caspase 9 [40]. The activated complex containing cytochrome C, Apaf-1 and caspase 9 is called the apoptosome and initiates cell death. In several cell types, the pro-apoptotic Bcl-2 family member Bim on mitochondrial membranes is involved in TCR-induced apoptosis. Bim is upregulated by p38 and JNK and causes mitochondrial membrane depolarization and thereby initiates the intrinsic death pathway (Figure 1) [41]. Bim is a critical initiator of mitochondria-dependent, stress-induced cell death and T cell homeostasis and serves as a barrier against autoimmune disease. Bim-deficiency severely impairs the apoptosis of autoreactive thymocytes [42].

Pro-apoptotic pathways are antagonized by anti-apoptotic vigors to ensure that apoptosis only occurs in response to appropriate death signals. So-called inhibitors of apoptosis (IAP) belong to this class of intrinsic proteins and a prominent member is the X-linked IAP (XIAP). IAP is able to set a threshold for the induction of apoptosis and inhibits effector caspases from becoming activated (Figure 1) [43, 44].

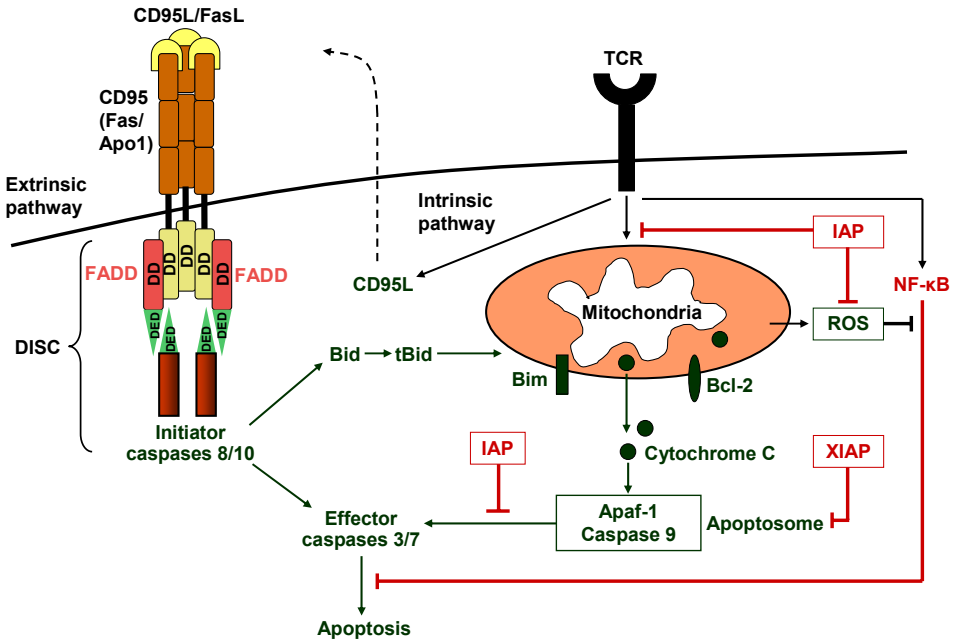

Figure 1. The extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is initiated by ligation of membrane-bound trimeric death receptors such as CD95/Fas. Adaptor molecules activate initator caspases through the DISC. The intrinsic pathway is induced by TCR stimulation and mitochondira membrane permeabilization.

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Apoptosis is also controlled by the transcription factor nuclear factor κB (NF-κB) pathway. During T cell development, activation of NF-κB provides a strong signal for cell survival and proliferation in response to TCR stimulation [45]. NF-κB is a negative regulator of PCD also in response to TNF-α and similar cytokines [46]. On the other hand, inhibition of NF-κB has a pro-apoptotic effect, particularly increasing the susceptibility to TNF-α-induced death by the increase of reactive oxygen species (ROS) [47, 48]. Increased ROS suppress Bcl-2 expression and results in cell death via the intrinsic apoptosis pathway [49].

T cell deletion in the thymus

T cell deletion works at two main stages: in the thymus during central tolerance and in the periphery following an immune response. Differentiation of thymocytes is characterized by the cell surface expression of proteins, such as CD4 and CD8. Bone marrow progenitors that enter the thymus initially do not express CD4 or CD8 and are referred to as double negative (DN) $CD4^-CD8^-$ thymocytes. Following T cell receptor (TCR) β chain rearrangement, only thymocytes expressing a functionally rearranged TCRβ chain are selected to continue maturation and upregulate both CD4 and CD8. At this $CD4^+CD8^+$-double positive (DP) stage, TCRα chain rearrangement is initiated and functionally rearranged TCRα chains are expressed on the cell surface together with the TCRβ chain to obtain a functional TCRαβ for recognizing antigens. Interactions between the TCR expressed by DP thymocytes and self-MHC molecules mediate survival or death. Thymocytes expressing TCR that do not interact with self-MHC molecules die within a few days. Thymocytes expressing TCRs that are able to interact with intermediate affinity with self-MHC molecules are rescued from death by a process called positive selection. Positive selection ensures that only T cells that are able to recognize peptides presented on self-MHC molecules are exported to the periphery. Thymocytes expressing TCRs recognizing class I MHC molecules downregulate CD4 and become cytotoxic $CD8^+$ single positive (SP) cells, while $CD4^+$ helper T cells arise as a result of interactions between TCR and class II MHC molecules. A strong interaction between TCR and self-peptide/MHC leads to the elimination of thymocytes by a process called negative selection [50, 51].

What happens to autoreactive thymocytes when they receive a strong signal through the TCR? Clonal deletion has been shown to be one of the main mechanisms that eliminates autoreactive cells [52-54]. Further evidence for clonal deletion as a mechanism of negative selection was provided by TCR transgenic mice [55, 56]. Together, these studies clearly demonstrate that thymocytes expressing self-reactive TCRs are clonally eliminated during development.

A potential problem with central tolerance is the limited expression of peripheral/tissue specific antigens in the thymus. It has become clear, however, that certain cells of the thymic stroma express peripheral tissue specific antigens that promote the elimination of self-reactive T cells [57, 58]. Studies have suggested that a putative transcription factor, the autoimmune regulator (AIRE) protein, is expressed in rare specialized cells called medullary epithelial cells (MEC). A mutation in this protein in human patients may lead to the development of the multiorgan autoimmune endocrine disease APECED [59, 60]. Since both AIRE and peripheral antigens are expressed in MEC, it was speculated and later confirmed by microarray technology, that AIRE controls the expression of these antigens [61]. To study the role of AIRE, AIRE-deficient mice were generated by two independent groups [61, 62]. These mice developed lymphocytic infiltrates and autoantibodies directed against a number of peripheral organs and tissues, such as the salivary gland, retina, pancreas, ovary, stomach and thyroid. Therefore, central tolerance includes mechanisms to induce tolerance of T cells specific for tissue antigens expressed outside the thymus.

T cell deletion to yield peripheral tolerance

Because not all autoreactive thymocytes are deleted during development, peripheral tolerance mechanisms exist to limit autoimmunity. Deletion of autoreactive T cells is one of these mechanisms. The mechanism of tolerance of mature T cells by clonal elimination was demonstrated *in vivo* using different approaches [63-65]. In the first report, injection of cells expressing the superantigen Mls-1^a into thymectomized Mls-1^b mice resulted in the expansion of Mls-1^a reactive $V\beta6^+CD4^+$ T cells, followed by deletion of these cells [63]. Further demonstrations of clonal deletion as a mechanism of peripheral tolerance to self-antigens was provided using transgenic mouse models [66-68].

The importance of deletion as a mechanism of peripheral tolerance has also been suggested in mice deficient for genes involved in apoptosis such as Fas and FasL. The natural mutant *lpr* mouse (lymphoproliferative), which carries a mutation in the TNF-family receptor Fas and the FasL mutant *gld* mouse (generalized lymphoproliferative disorder), develop lymphadenopathy, splenomegaly and spontaneous autoimmunity [69, 70]. A similar phenotype and autoimmunity is seen in

autoimmune lymphoproliferative syndrome (ALPS) patients (also known as Canale Smith Syndrome), most of whom have a mutation in Fas [71-73]. However, the role of Fas in peripheral clonal deletion and autoimmunity remains controversial. While some studies have pointed to a role for Fas/FasL in AICD of mature T cells but not thymocytes [74, 75], other studies found Fas to be dispensable for peripheral deletion [76-80]. Numerous other examples of molecules involved in apoptosis exist whose deregulation can result in autoimmunity [81-83].

Defects in T cell apoptosis in T1DM

Similarly, defects in apoptosis have also been suggested in diabetes. Studies using NOD mice have shown that normal thymic negative selection in these mice is impaired. It has been suggested that in NOD thymocytes the FLICE-inhibitory protein is upregulated upon TCR engagement which may result in the prevention of normal T cell deletion [20]. A further study using the BDC2.5 transgenic T cells on a NOD background suggests that a number of other apoptotic genes are differentially upregulated in NOD thymocytes during negative selection. Interestingly, on the NOD background, a different set of genes was up- or downregulated, rather than the same genes to different degrees. For example, the anti-apoptotic gene *bcl2* was increased more than 2-fold on the NOD background compared to controls [84]. The authors then located *Idd* loci and genes responsible for the negative selection defect. It turned out that, besides *bcl2*, PD1 (another protein involved in apoptosis) was also implicated in the control of immune tolerance. Although very interesting, neither of the above-mentioned studies proved a causal relationship that impaired negative selection is the trigger or cause of diabetes in NOD mice.

Some studies have also suggested defects in peripheral AICD that follows immune responses [85, 86]. Lower levels and/or activity of caspase 8, as well as Fas/FasL were suggested as reasons for this difference. Furthermore, c-FLIP expression persists in NOD T cells. In general, there is a limited amount of evidence for abnormal T cell apoptosis in the literature. It is possible that the resistance to AICD arises from the defective proliferative response of NOD T cells, which can be overcome by IL-4 [22]. Similarly, the low level of FasL in NOD mice does not correlate with studies carried out in humans where no linkage of polymorphisms in the *fasl* gene to diabetes were found [87].

Overcoming defects in T cell apoptosis in order to reverse T1DM

How could potentially impaired T cell apoptosis processes in diabetes be reversed? This may be achieved either directly or indirectly. For example, injection of non-specific agents such as BCG or complete Freund's adjuvant (CFA) prevents diabetes in NOD mice [88]. In an experiment carried out by Qin and coworkers, BCG immunization induced the production of TNF-α, IFN-γ and IL-4 by splenocytes, while, at the same time, it increased the expression of Fas, FasL and TNFR on T cells resulting in their apoptosis. The same effect could be reproduced by the systemic administration of the Th1 cytokines in these mice [88]. Problematically, such systemic treatments reduce the number of T cells of all specificities, not just the autoimmune ones, and may potentially leave the organism more susceptible to side effects such as infections or tumors.

A more specific or direct approach may involve increasing the concentration of negatively selecting peptides in the thymus. Peptides directly involved in the activation of autoreactive T cells (insulin, GAD, IA-2, ICA512) may be expressed at higher concentrations in both the thymus and the periphery. However, at this time, the technology available to achieve this effectively is limited and/or yet to be shown as being safe. Furthermore, this strategy can only be considered in subjects susceptible to develop diabetes and not in patients who have already developed the disease.

In cases of specific overabundance of anti-apoptotic factors (e.g. Bcl-2 in NOD mice, as discussed above), molecules could be developed that would target and block the function of these anti-apoptotic molecules. This is currently being tested in cancer patients [89]. However, the side effects may be still too great to justify the use of such compounds in diabetes patients. Hence, highly specific inhibitors need to be developed. An example of a more physiologic compound is vitamin D which has also been shown to work in NOD mice in preventing diabetes [90]. Although its mechanism of action is unclear, vitamin D enhances the susceptibility of thymocytes to apoptosis. Whether this approach would also be applicable in humans remains to be determined.

Although interesting and promising, many of the abovementioned treatments may be difficult in humans, as currently, there is no clear way of determining who will develop diabetes in the future and for whom such immunomodulatory techniques would be useful until clinical disease develops. However, because clini-

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cal diabetes is believed to occur when more than 90% of β-cells have been destroyed, it leaves a small window to counteract and potentially reverse the autoimmune process. These techniques may also be useful in the future in cases of autologous islet transplants from, for example, stem cell sources where the immune system would still be potentially autoreactive towards β-cells and tolerizing regimens would be necessary to prevent a recurrence of diabetes.

Conclusion

Apoptosis is crucial for the maintenance of tolerance and the prevention of autoimmunity. However, as of today, there is limited evidence to suggest that faulty apoptotic mechanisms are responsible for the development of diabetes. Although the evidence for a negative selection defect in the thymus is stronger than for one in the periphery, diabetes appears to be a more complicated disease involving a multitude of factors. Some of these may be components of the apoptotic machinery and would thus be interesting to investigate in the future. Thus, further studies will have to delineate apoptotic defects and to associate them with diabetes development, before potential treatment approaches can be considered.

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