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Apoptosis and the Developing T Cells

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Abstract

Efficient TCR repertoire selection in the thymus is critical for immune function, ensuring the production of functional MHC-restricted and self-tolerant T cells. T cell education in the thymus involves positive and negative selection processes where apoptosis play an especially important role in eliminating useless or potentially dangerous thymocytes. For decades, positive and negative selection in T cell development has attracted the attention and considerable research has been conducted to improve our understanding of how ligand induced signaling through the T cell receptor (TCR) can lead to both: rescue from death in the case of positive selection and death in the case of negative selection. In this brief report, we review the basic concepts involved in the extrinsic and intrinsic pathway of apoptosis, and provide an overview of the events that leads immature T cells to survive or die by apoptosis during their intrathymic development.

Keywords: T cell development; Thymocyte; Programmed cell death; β selection; Positive selection; Negative selection

Introduction

T cells or T lymphocytes play a central role in immune response. They are generated from bone marrow-derived lymphocyte precursors that enter the thymic gland through blood vessels. Currently, little is known of the mechanisms that attract precursors to the thymus or facilitate their migration through the surrounding tissues. Also, the phenotype of Tcell precursors remains unclear but some markers, such as c-Kit (CD117), CD25, and CD34 have been reported to be associated with them [1]. In addition, the rationale for this ectopic delivery system has never been elucidated, although one thing is clear, it is not a steady-state process.

The thymus, a primary lymphoid organ, plays a crucial role in the development of T cells providing an inductive microenvironment in which committed progenitors interact with stromal cells (and their soluble products) and extracellular matrix proteins to receive appropriate signals for survival, proliferation and differentiation [2,3]. Once in the thymus, the T cell precursors (or thymocytes, while in the thymus) undergo selection processes that lead to generation of mature T cells [4]. These mature T cells, which are immunocompetent resting cells, leave the thymus to populate the peripheral or secondary lymphoid tissues. Those thymocytes that are not selected die by genetically programmed cell suicide, a process known as apoptosis, and are phagocytized by thymic macrophages.

Apoptosis, or programmed cell death, is a process by which cells play an active role in their own death and undergo organized self-destruction without eliciting an inflammatory response. This cell death process plays significant role in development and aging, tissue homeostasis, in response to a variety of physiological and pathophysiological stimuli, and also in the selective deletion of developing T cells during their intrathymic journey.

Apoptosis Activation Pathways

Apoptosis can be mediated by different mechanisms and several stimuli, which may originate either extracellularly (the extrinsic apoptotic pathway) or intracellularly (the intrinsic apoptotic pathway), may trigger the process. The extrinsic pathway is activated from outside the cell by pro-apoptotic ligands that interact with specialized cell surface molecules, termed death receptors (DRs). The intrinsic pathway is activated from inside the cell by pro-apoptotic and anti-apoptotic members of the B-cell leukemia/lymphoma 2 (Bcl-2) protein family and other mitochondrial molecules, such as hydrogen peroxide. Both pathways of apoptosis activation, however, converge to the same effector mechanism whose components belong to a family of cysteine proteases called caspases, which carries out important proteolytic events that breakdown structural component of the cell leading to partition of nucleus and cytoplasm into membrane bound-apoptotic bodies [for review, 5]. Unlike another form of cell death called necrosis, there is no inflammatory response during apoptosis process since cell fragments are quickly removed from the microenvironment by neighboring phagocytic cells.

The extrinsic or death receptor pathway of apoptosis

In the extrinsic apoptotic pathway, the caspase cascade is triggered by the activation of DRs on the cell surface. DRs are members of the tumor necrosis factor receptor (TNFR) superfamily, which includes: CD95 (Fas/Apo1) [6], TNF receptor 1 (TNFR-1/p55) [7], TNF receptor superfamily, member 25 (TNFRS25/TRAMP/WSL-1/Apo3/DR3/LARD) [8], TNF-related apoptosis-inducing ligand– receptor 1 (TRAIL-R1/DR4) [9],TRAIL-R2 (DR5/Apo2/KILLER) [10] and TNF receptor superfamily, member 21 (TNFRSF21/DR6) [11].The ligands for DRs are Fas Ligand (CD95L) that binds CD95, TNF and lymphotoxin α, which bind to TNFR1 [12], TNF-like weak inducer of apoptosis (TWEAK/Apo3 ligand) that binds to TNFRS25 [13] and TNF-related apoptosis-inducing ligand (TRAIL/Apo2 ligand) that is the ligand for both TRAIL-R1 [9] and TRAIL-R2 [14]. Until now, DR6 is an orphan TNF receptor superfamily member and its role as an apoptosis-inducing receptor is less clear and perhaps cell type dependent [11].

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CD95 is the best-characterized member of the TNFR superfamily and has served as a prototype because its role in lymphocyte regulation is better established. CD95 receptors are expressed on the surface of cells as preassociated homotrimers and are characterized by the presence of a cytoplasmic region termed the death domain (DD) [15]. CD95L, the ligand for CD95 is typically found in the plasma membrane of cells and like CD95 is expressed as homotrimers. Although constitutive CD95L expression is observed in some immune privileged tissues such as eye and testes, CD95L's up regulation on T lymphocytes requires their activation by ligation of the T cell receptor (TCR). CD95L can induce apoptosis both in an autocrine (suicide) or paracrine (fratricide) CD95 fashion, which means that CD95L can engage CD95 either on the same cell or on another cell [16]. CD95L is involved in the induction of cell death known as activation-induced cell death (AICD) because it is induced by lymphocyte activation and not by the absence of stimuli [17]. This process is highly needed to prevent an excessive immune response and eliminate autoreactive Tcells in the peripheral lymphoid organs.

In response to CD95L, CD95 recruits, through homotypic interaction of their DD, an adaptor molecule called Fas-associated death domain (FADD) [18]. In its N-terminal region, FADD contains a "death effector domain" (DED) that is responsible for engagement of at least two members of the caspase family caspase-8 and -10, which also display these DED domains. Both the DD and DED enable proteins containing the same domains to interact with one another. Besides, an inactive homologue of caspase 8, named cellular caspase inhibitor FLICE-like inhibitory protein (cFLIP), which is predominantly expressed in muscle and lymphoid tissues, can be recruited to DR following ligand binding via the adaptor molecule FADD [19]. Elevated levels of FLIP can displace caspase-8 from the activated DR complex, acting as a dominant inhibitor of caspase-8 and thereby preventing the activation of distal caspases and cell death [20]. Although originally identified in viral DNA as an inhibitor of DR signaling, literature has shown that cFLIP (along with caspase-8) is required for survival and proliferation of T cells, both in thymocyte development and after lymphocyte activation [21,22,23]. This set of proteins, *i.e.* the trimerized DR; the adaptor molecule FADD; the procaspase-8 and -10; and cFLIP, forms a large complex of proteins called the death-inducing signaling complex (DISC) that is essential for induction of apoptosis [24].

The binding of procaspase-8 and -10 to the DISC results in processing of the zymogen, and as a result the active caspase-8 heterotetramer (containing two small and two large subunits) is released into the cytosol to propagate the apoptotic signal [25]. At present, our understanding on the activation of initiator caspases is very limited. The Induced Proximity model, first proposed in 1998, states that the initiator caspases autoprocess themselves when brought into close proximity of each other [26]. This model was further reinterpreted to be proximity-driven dimerization of initiator caspases, and consequently their activation [27]. It is still controversial whether caspase-10 can trigger cell death in the absence of caspase-8, since the expression levels of pro-caspase-10 in many cells are probably not high enough to launch apoptosis alone [28].

It has been established two CD95 signaling pathways [29]. Type I cells are characterized by high levels of CD95 DISC formation and increased amounts of active caspase-8. Activated caspase-8 directly leads to the activation of downstream effector caspase-3, -6 and -7. Type II cells are characterized by lower levels of CD95 DISC formation and, thus, lower levels of active caspase-8. In this case, signaling requires an additional amplification loop that involves the cleavage by caspase-8 of a member of the Bcl-2-family named protein Bid (BH3-interacting domain death agonist) to generate truncated Bid (tBID). This fragment induces the proapoptotic functions of the mitochondria by causing aggregation of Bax (Bcl-2-associated X protein) or Bak (Bcl-2 antagonist/killer) and subsequent release of *cytochrome c* from mitochondria [30]. Once *cytochrome c* is released, it binds and activates the cytosolic protein Apaf-1 (Apoptotic protease activating factor 1) to facilitate the formation of the adaptor protein complex, named apoptosome, which mediates the activation of the initiator procaspase-9 [31]. Like caspase-8, caspase-9 can directly activate the effector caspases. Expression of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x, can block the CD95-mediated apoptosis in type II cells [29]. It appears likely, that the central function of mammalian Bcl-2 family members is to guard mitochondrial integrity and to control the release of mitochondrial proteins into the cytoplasm [32]. Accordingly, antiapoptotic Bcl-2 members sequester proapoptotic Bcl-2 members by binding to their BH3 domains and thereby ultimately prevent Bax or Bak activation/oligomerization and consequently inhibit mitochondrial proapoptotic events [33]. Alternatively, Bcl-2 also appears to inhibit apoptotic pathways that are independent of Apaf-1/caspase-9 and which might depend on caspase-7 as a central effector [34].

The role of initiator caspase-2 in DR signaling remains contradictory. Caspase-2 was shown to be processed in the course of TNFR1-mediated apoptosis [35]. In response to its ligands (TNF and lymphotoxin α), TNFR-1 is trimerized and recruit TNFR-associated death domain protein (TRADD) as adaptor molecules [36]. As TRADD does not contain a DED region, it works by binding to FADD via interactions between their death domains. So, both CD95 and TNFR-1 DRs use FADD as adaptor molecule to mediate cell apoptotic signals. TNFR-1, however, can use another pathway to transduce the signal for apoptosis by using the molecule termed receptor interacting protein (RIP), which interacts with TRADD via interactions between their death domains [37]. But like TRADD, RIP does not carry a death effector domain and another downstream effector molecule, named RIP associated ICH1/CED3 homologous protein with death domain (RAIDD). RAIDD contains both death domains (DD) and caspase recruitment domains (CARD) [38]. As interactions between the molecules at the DISC are based on the contacts between homotypic domains, RAIDD specifically binds RIP, through DD, and then recruits caspase-2 to the TNF receptor signaling complex, through CARD. The presence and the activation of procaspase-2 at CD95 DISC were reported in human T- and B-cell lines. However, it was also shown that procaspase-2 in the absence of procaspase-8 does not initiate CD95-induced apoptosis [39]. Therefore, the exact role of procapase-2 in CD95-mediated apoptosis remains a matter for future studies.

The intrinsic or mitochondrial pathway of apoptosis

The intrinsic pathway of apoptosis is also called "death by neglect" or "activated cell autonomous death" (ACAD), and it does not require signals resulting from the engagement of DRs. This pathway of apoptosis can be triggered by many stimuli such as TCR stimulation, absence of survival signals, DNA damage, oxidative stress, cytokine and costimulators deprivation, viral infection, endoplasmic reticulum stress, as well as those induced by chemotherapeutic drugs [40,41]. Mitochondria play a pivotal role in this form of apoptosis [42,43]. In this apoptotic signaling pathway, pro- and anti-apoptotic members of Bcl-2 family play important role in the mediation and regulation of

cell death. The current model of how Bcl-2 family members regulate apoptosis describe that upon stress-apoptotic stimuli Bcl-2-interacting mediator of cell death (Bim) or p53-upregulated modulator of apoptosis (PUMA) proteins are activated and hence displace anti-apoptotic Bcl-2 family members on the outer mitochondrial membrane, resulting in the release of Bax- and/or Bak-like pro-apoptotic factors. Bax- and/or Bak-like factors undergo a conformational change, insert into the outer mitochondrial membrane and provoke a sudden increase of the inner mitochondrial membrane permeability, the so called permeability transition (PT). This event leads to the release of pro-apoptotic proteins, including *cytochrome c*, Smac/DIABLO, and the serine protease HtrA2/Omi [44-47] into the cytoplasm [48]. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAP) activity [46,49]. Mitochondria also release apoptosis-inducing factor (AIF) and endonuclease G, which appear to kill independently of caspases [50,51].

Like in the extrinsic pathway of apoptosis, the release of *cytochrome c* from mitochondria results in apoptosome formation followed by activation of procaspase-9, which in turn cleaves downstream effectors caspase-3, caspase-6 and caspase-7 [31].

Although apoptosome mediated caspase-9 activation is widely accepted as the initiating event in the intrinsic pathway of apoptosis, several other studies suggest that an initial caspase activation occurs upstream of the mitochondria and is required for mitochondrial permeabilisation [34,52,53,54]. The work by Lassus and his group demonstrates that one potential candidate working upstream of mitochondria is caspase-2 [52]. So, according to these works, in the intrinsic pathway of cell death, mitochondria can act only as amplifiers of caspase activity rather than initiator of caspase activation as occurs in the extrinsic pathway of apoptosis.

Apoptosis effector mechanisms: the caspases

As seen above, the two distinct pathways of apoptosis lead to the activation of effector caspases, which carry out the death signal through cleavage of many different cellular protein substrates vital for cell functions.

Caspase-3 is considered to be the most important of the executioner caspases. Once activated, caspase 3 cleaves several substrates, including the actin, intermediate filament proteins, the nuclear/mitotic apparatus protein NuMA, and cytokeratins [55,56].The caspase-mediated cleavages of these structures, which are responsible for the maintenance of cell, contribute to apoptotic morphological changes often observed during apoptosis. Several biochemical changes observed in apoptotic cells also result from caspase-induced cleavages. Caspase 3 cleaves the nuclease inhibitor ICAD (inhibitor of caspase-activated deoxyribonuclease), allowing subsequent internucleosomal cleavage of DNAby the constitutively expressed nuclear enzyme CAD [57-60]. In addition, caspase-3 cleaves many protein kinases whose activation leads to presentation of a variety of intracellular molecules on the cell surface that are recognized by receptors on the cell surface of the macrophages [61-65].

Caspase-3 also cleaves caspase-6, which in turn is activated. Caspase-6 appears to be uniquely capable of cleaving Lamin A in nuclei that contain this particular intermediate filament protein [66,67].

Caspase-7 is cleaved by caspase-3, caspase-9 and caspase-10, and, several substrates that are efficiently cleaved by caspase-3 can also be targeted by caspase-7, suggesting an at least partial redundancy of both caspases.

Upon activation, the caspases cause the morphological and biochemical changes characteristic of apoptosis, such as nuclear membrane breakdown, chromatin condensation, chromosomal DNA fragmentation, and the formation of apoptotic bodies. At last, the apoptotic cells are efficiently phagocytized by neighboring cells without an inflammatory response.

T Cell Development in the Thymus

The development of Tcells within the thymus is a complex process that involves several stages based on their expression of CD4 and CD8 co-receptors. At early stage of development, Tcell precursors have a $CD4^-CD8^-$ "double negative" (DN) phenotype. This phase is also characterized by differential expression of the CD44 and CD25 molecules, which define four differentiation stages of DN thymocytes, with the developmental progression being $CD44^+/CD25^-$ (DN1) to $CD44^+/CD25^+$ (DN2) to $CD25^+/CD44^-$ (DN3) and then to $CD44^-/CD25^-$ (DN4) cells [68,69].At this stage, thymocytes begin to rearrange and express their TCR β, γ and δ genes and the two lineages of T cell (αβ and γδ) also begin to diverge at this point [70]. Among the two types of T cells, αβ T cells are the most abundant and therefore we will focus only on αβ-expressing thymocytes development.

A first step toward the expression of a functional Tcell receptor (TCR) takes place since the ß locus of the TCR is rearranged and tested for functionality by pairing to the pre-TCRα-chain. In the case of productive ß rearrangement and successful formation of the pre-TCR complex (β/pTα), thymocytes enter the cell cycle to expand, down-regulate CD25, and develop into $CD44^-CD25^-$ (DN4) cells [71]. Pre-TCR signaling confers survival and allows development to proceed through a $CD4^+CD8^+TCR^{low}$ double-positive (DP) subset of thymocytes, which constitute the vast majority of thymocytes since they represent about 80% of the total cells in the organ. The interaction with low affinity between the TCR and endogenous peptides presented by self-major histocompatibility complex (self-pMHC) expressed on epithelial cells in the thymic cortex will determine the positive selection of DP thymocytes by delivery of survival and differentiation signals. DP thymocytes that fail to engage self-pMHC die by apoptosis because they do not receive a survival signal. Positively selected DP thymocytes mature into $TCR^+CD4^+CD8^-$ and $TCR^+CD4^-CD8^+$ single-positive (SP) cells, and migrate into the thymic medulla where they undergo negative selection. During this process, thymocytes that are not strongly activated by self-pMHC are allowed to survival and emigrate to peripheral lymphoid organs as mature T cells, naïve T helper cells (CD4) or cytotoxic T cells (CD8). In contrast, thymocytes reacting strongly to self-pMHC are eliminated by apoptosis.

So, programmed cell death during thymocyte development is used to eliminate useless precursor cells with non-rearranged or aberrantly rearranged non-functional antigen receptors. In addition, apoptosis is essential for deletion of auto reactive T cells in the thymus. This mechanism is the basis of central self-tolerance.

The intrathymic journey of thymocytes: a time to live or a time to die?

The thymus gland, located in the anterior mediastinum, consists of two encapsulated lobes that are divided by numerous septa into multiple lobules. Each lobule presents two different regions, i.e., cortex and medulla. The outer cortical portion is densely populated by the

least mature thymocytes and the inner medullary portion contains few, but fully mature, T cells. The thymic environment is formed by epithelial cells, which form a meshwork to provide mechanical support and stimuli for the proliferation and development of thymocytes, and by macrophages, dendritic cells, fibroblasts and matrix molecules [72]. The successful development of mature T cells depends on the constant migration of the thymocytes through the thymic microenvironment. Such migration is essential for thymocytes receive signals from different thymic stromal cells leading to their proliferation, differentiation and generation of diversity [73]. Although the mechanisms directing this migration is poorly understood, clear evidence has been obtained showing that thymic microenvironment, collectively, influences the process of thymocyte maturation through surface molecules and by secreting soluble polypeptides as cytokines (especially IL-7), chemokines (like CXCL-12, expressed by stromal cells in the cortex) and hormones.

Apoptosis in DN thymocytes

DN thymocytes, also called pro-Tcell, are located in the outermost cortex, which is the thymic zone where the rate of cell proliferation and death by apoptosis are extremely high. For thymocytes at this stage of maturation, the cytokine IL-7, also known as lymphopoietic cytokine, has a critical role in controlling life and death decisions. IL-7, one of the most important cytokines in thymus, is constitutively produced by stromal cells and plays a crucial role in thymopoiesis since it sustains thymocyte proliferation and survival [74]. Once presenting DN1 phenotype, thymocytes needs IL-7 stimulus to survive and progress to DN2 stage [75]. The role of IL-7, as well as its cognitive receptor (IL-7R), at the DN1-DN2 transition involves up-regulation of the anti-apoptotic proteins, bcl-2 and Mcl-1(myeloid cell leukemia 1), which can act as an apical molecule in apoptosis control, promoting cell survival by interfering at an early stage in a cascade of events leading to release of *cytochrome c* from mitochondria [76]. Experiments using IL-7- or IL-7R-deficient mice show reduced numbers of thymocytes and no progress from DN1 to DN2 stage, confirming that the pro-survival signaling from IL-7/IL7R is vital for these cell subsets [77-79].

At the DN3/DN4 stage, proteins produced from productively rearranged TCRβ genes must be assembled into the pre-TCR complex, which consists of a TCRβ-chain, the invariant pTα-chain, and proteins of the CD3 receptor complex [80]. Only thymocytes that have a functional pre-TCR survive the transition from DN4 to DP, a process also known as β selection.

Thymocytes that pass through the β-selection step represent the DN4 stage, or the pre-double positive (DP) stage of development. One of the effects of pre-TCR signaling is the inactivation of tumor suppressor p53, thus ensuring survival of ß-selected cells and the release of the cell cycle block, allowing for the proliferative burst observed between the DN3 and DP stages [81]. Although pre-TCR signals is the most important survival signaling only pre-TCR signaling is not sufficiently to maintain DN3/DN4 thymocytes alive as well as to support its differentiation to DP stage. So, other receptors, including members of the Notch signaling molecules family, and the CXC chemokine receptor 4 (CXCR4), are essential to the complete T cell development [82-84]. Among the four known Notch receptors, Notch1 has been shown to be a critical component in the process of T cell development [85]. Notch1 is expressed at relatively high levels in the DN thymocytes (least mature cells) and at very low levels in mature single positive ($CD4^+CD8^-$ and $CD4^-CD8+$) cells, an expression pattern consistent with a role for Notch in maintaining cells in a less differentiated state [86,87]. The Notch activity is mediated by cyclin dependent kinase-6 (CDK6) that contributes to the Notch receptor signaling as well as to the expression of its target genes. In the absence of CDK6, the Notch signaling pathway is deficient and fall of thymic cellularity is observed in all stages of differentiation [88]. Recent studies have demonstrated that inactivation of Notch1 at DN2-DN3 stage induces DN3 thymocytes accumulation as a consequence of impairment in Vβ to DJβ rearrangement [89,90]. Furthermore, Ciofani and colleagues have shown the cooperation between Notch and pre-TCR signals during β-selection process by using an *in vitro* model for T cell development, the Notch delta-like-1 ligand-expressing OP9-DL1 stromal cells [91]. Also recently, Trampont and Janas, along with their collaborators, have demonstrated an important role for CXCR4, which is expressed by all early progenitors in the thymus, and its ligand, CXCL-12, in β-selection of thymocytes [92,93].

Stimulation either by Notch or CXCR4 receptors leads to the protein kinases AKT (also known as protein kinase B) phosphorylation through the phosphatidylinositol 3-kinase (PI3K). Via directly phosphorylating several substrates, AKT plays a central role in promoting cell survival and proliferation [94]. In the absence of AKT, DN3 thymocytes undergo apoptosis due to reduced expression of essential nutrient receptors, like the transferrin receptor protein 1 (TfR1) also known as CD71), which depends on signals transmitted by this kinase to be expressed on cell surface [95,96].

Soon after the expression and signaling by pre-TCR molecules, genes encoding the TCR α chain are rearranged and both chains of αβTCR are expressed on the cell surface in association with CD3 and ζ proteins. Then, DN4 thymocytes undergo proliferation and migrate toward the inner cortex while up-regulating CD4 and CD8 molecules to become double-positive DP cells ($CD4^+CD8^+$). The function of CD4 and CD8 molecules is to facilitate the interaction of the TCR with, respectively, non-polymorphic portions of class II MHC and class I MHC that are expressed on antigen presenting cell (APCs), including cortical and medullary thymic epithelial cells, and assist the TCR in binding and possibly in signaling.

Life and death during positive selection

DP thymocytes that have productively rearranged their genes and express a complete and functional αβTCRs are tested by the process named positive selection. So, DP thymocytes encounter, in the inner cortex, thymic epithelial cells that are displaying self-peptides bound to class I and class II MHC molecules. The goal of this selection is the generation of an immune system that is capable of recognizing a large number of antigens and of discriminating between self and non-self-antigens.

The difficulty with positive selection is to explain how the TCR can engage self-MHC without stimulating apoptotic mechanisms required to avoid auto reactivity. One widely accepted model has been proposed to address this question: the "strength of signaling" hypothesis, proposing that quantitative attributes of TCR signaling instruct cell fate during thymocyte development [97]. Accordingly, relatively rare, low-affinity self-peptides presented by self-MHC induce survival and differentiation, allowing positive selection of DP thymocytes. It is believed that through this mechanism mature T cells whose precursors were positively selected by self-MHC will be able to recognize foreign antigens, which are generally structurally related to the self-peptides involved in thymic selection, displayed by the same MHC molecule

expressed on peripheral APCs. On the other hand, abundant, high avidity/affinity self-peptides induce clonal deletion [reviewed in 98].

The role of several intracellular mediators of activation has been extensively studied and none of them is exclusively associated with survival or death in developing thymocyte. Evidences have yet been demonstrated that mitogen-activated protein kinase (MAPK) cascades are activated after TCR/pMHC interaction to determine the fate of developing thymocyte [99-101]. The fourteen known MAPK isoforms that have been identified in mammalian cells can be divided into four groups: the classical MAPK (ERK1/2), p38 MAPK, c-Jun N-terminal kinase (JNK) and the atypical MAPK, which include ERK5, ERK3 and ERK8 [102-105]. Although the proximal TCR signaling components Lck and ZAP70 are activated similarly by both positively and negatively selecting ligands, the linker for activation of T cells (LAT), an adapter protein that couples the antigen receptor to downstream signaling pathways, is able to specifically recruit different signaling molecules that are essential for thymocyte selection [reviewed in 106]. Therefore, LAT may be the initial molecule responsible for triggering different signals in response to positive and negative ligands. The interactions between the TCR and the ligand are propagated through different downstream events: at least through Grb2 and RasGRP1/MAPK pathways [reviewed in 107]. It has been suggested that positively selecting ligands induce partial phosphorylation of LAT, which recruit few molecules of Grb2 to the Golgi with only ERK1/2 activation, and cell survival. In contrast, negatively selecting ligands induce strong antigen receptor signals that recruit numerous Grb2 adaptor proteins to the plasma membrane with ERK1/2, p38, and JNK activation, and apoptosis [108-110].

In response to weak/moderate TCR signaling DP thymocytes can be induced to undergo maturation by the GTPase Ras pathway that leads to Raf1–MEK1⁄2–ERK1⁄ 2 activation, which in turn activates several transcription factors [111]. The Early growth response gene 1 (Egr1) has been proposed as one of the earliest transcription factors expressed after TCR stimulation on DP thymocytes [112,113]. Bettini and collaborators, by using Egr1-deficient mice, have demonstrated a role for Egr1 in enhancing the expression of negative regulators of differentiation pathways, like the Inhibitor of differentiation/ DNA binding type 3 (Id3), and the anti-apoptosis molecule Bcl-2 [114]. Recently, Lauritsen and co-workers have reported that another transcription factor, Erg-2, has a central role during positive selection since it up regulates the survival molecule Bcl-2 [115]. Other transcriptional factors, like retinoid-related orphan receptor gamma (RORγ) and T-cell factor-1 (TCF-1), and more recently the cMyb, have also been implicated during positive selection. Yuan and colleagues demonstrate that in the absence of cMyb DP thymocytes die due to the under expression of the anti-apoptotic molecule Bcl-xl [116]. Other authors have also reported that up regulation of the anti-apoptotic Bcl-xl molecule is a key event to thymocyte survival at DP stage [117]. Recent experiments have determined a complex and unique role of the BCL11B transcription factor in the control of both positive selection and survival of DP thymocytes [118]. In addition, ERK1⁄ 2 may also

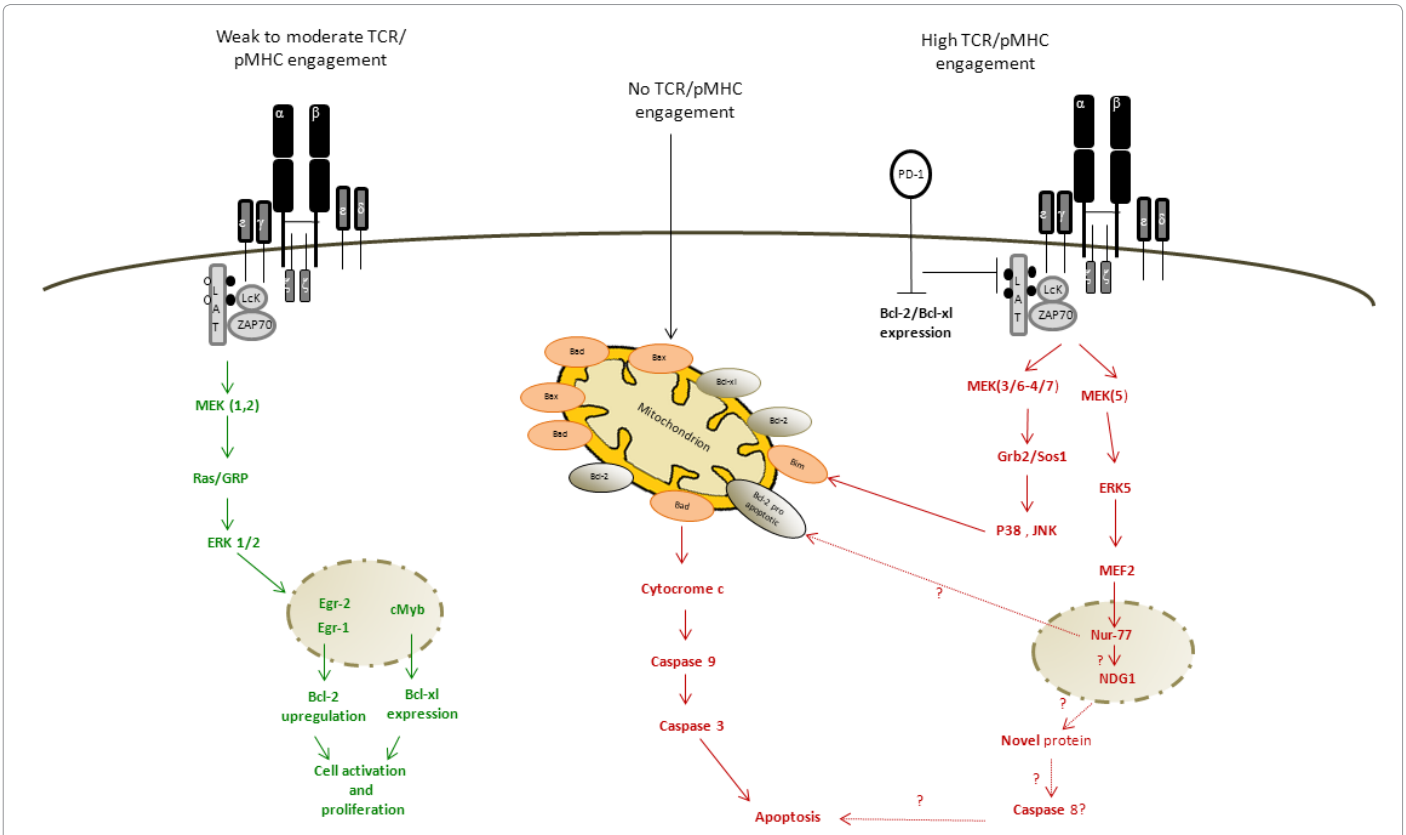

Figure 1: Simplified schematic representation of some intrathymic events that determine the developing T cell fate. In response to weak/moderate TCR signaling DP thymocytes can be induced to undergo maturation by the Ras pathway (left panel). The strong and prolonged interaction TCR/pMHC, with or without costimulatory molecules (represented by PD-1), will result in elimination of thymocytes by apoptosis (right panel). Thymocytes that fail to express a functional αβTCR cannot undergo maturation and they die due to lack of "survival signals" delivered throughout TCR (middle panel).

contribute to thymic selection by regulating the balance of pro- and anti-apoptotic proteins in the cytosol since ERK-mediated phosphorylation of Bim can target it for degradation or inhibit its pro-apoptotic activity by reducing its binding to the anti-apoptotic molecules Mcl-1 and Bcl-xl [119-121]. Although there are many different enzymatic pathways that activate thymocyte differentiation or death, a new report points the molecule identified as schnurri-2 (Shn2) as a crucial death dampener [122]. According to the authors, Shn2 functions downstream of TCR proximal signaling components to dampen Bax activation and the mitochondrial death pathway.

DP thymocytes that fail to express a functional αβTCR cannot undergo maturation and they die due to lack of "survival signals" delivered throughout TCR signaling. This "death by neglect" correlates with low expression of Bcl-xl and Bcl-2 survival factors and high expression of pro-apoptotic factors. Studies with thymocytes from animals that do not express Bak and Bax proteins have showed resistance to apoptosis, revealing that death in the thymic positive selection is dependent on the translocation of these pro-apoptotic factors from membrane to cytosol of mitochondria and the release of several apoptogenic factors including cytochrome c that leads to the activation of caspase-9 [123]. Other *in vitro* experiments using Bim-deficient thymocytes have reported resistance to apoptosis as well as the thymocytes that express anti-apoptotic proteins, such as Bcl-2, Bcl-xl and Mcl-1[124]. Recently, Ryan and colleagues have also demonstrated that mitochondria in DP thymocytes are more primed for death signals than mitochondria from other thymocytes, pointing to the BIM protein as the critical fator for this increased sensitivity [125].

It has also been proposed that glucocorticoids (GCs) are the major players in death by neglect, acting as regulators of the differentiation and selection of developing thymocytes at this stage of maturation [126-128]. In fact, DP thymocytes are the most sensitive thymic sub-population to GC-induced apoptosis [127].

Thymic stromal cells are known to produce GCs locally [129], and more recently, it was demonstrated that thymocytes can secrete GCs, too, in an age-dependent manner [130]. Therefore, thymocytes are located in a GC-rich microenvironment [131]. Until now, however, the higher susceptibility of DP thymocytes to apoptosis is not well understood. It has been shown that GCs control selection of DP thymocytes by modifying their TCR signal [132]. According to Talabér and colleagues the sensitivity of DP thymocytes to GC-induced apoptosis correlates with rapid mitochondrial Glucocorticoid receptor (GR) translocation upon ligand binding, which could initiate apoptotic pathways [133]. In a recent report, Xue and his group have demonstrated that the increased expression of cell cycle proteins in DP thymocytes contributes to their intrinsically sensitive to apoptosis [134].

A novel modulator of thymocyte GC-induced apoptosis, Murine SWI3-related gene (SRG3), has been suggested to play an important role in regulating GC-induce apoptosis of DP thymocyte [135]. Some experiments have shown a strict correlation in SRG3 expression and GC-induced apoptosis. DP thymocytes that express low levels of SRG3 in consequence of the TCR signaling and Notch activity present resistance to GC-induce apoptosis [136].Also, Jeong and his group have observed that Nitric Oxide (NO) may also inhibit GC-induced apoptosis of immature thymocytes by down-regulating the SRG3 expression [137].

So, during the positive selection process, thymocytes expressing useless or self-reactive TCRs are excluded by apoptosis from the T cell repertoire. On the other hand, this process also allows the positively selected thymocyte to survive and differentiate into $TCR^{+}CD4^{+}CD8^{-}$ and $TCR^{+}CD4^{-}CD8^{+}$ single-positive (SP) cells. Then, the thymocytes migrate into the thymic medulla, where they stay for as long as 10-14 days before emigrate to the T cell areas of the secondary lymphoid organs[138]. In the medulla, the functional maturation of thymocyte will be completed since they will interact with APCs that are responsible for the negative selection process.

Life and death during negative selection

Some of positively selectedSPthymocytesmay present TCRs with high affinity/avidity for non-thymic peptides (or "tissue-specific antigens") that are expressed at high concentrations on thymic medullary epithelial cells, dendritic cells and macrophages. The expression of such tissue-specific proteins is controlled by the autoimmune regulator (*AIRE*) gene [139]. The strong and prolonged interaction with MHC and self-peptide will result in elimination of thymocytes by apoptosis. This mechanism has particular importance in the removal of cells that could recognize self-antigens in the periphery by presenting auto reactive receptors. So, through the clonal deletion of thymic immature T cell displaying potential self-reactivity, the negative selection process ensures that only T cells that do not recognize self-antigens undergo their development.

Apoptosis during the negative selection process appears to share some of the pathways that are used by T cell activation, although the threshold for activation of thymocyte apoptosis during the negative selection process is lower than the threshold for activation of mature T cells.

Several lines of evidence indicate that costimulatory molecules may act together with TCR/CD3 complex to activate a pathway leading to programmed cell death of thymocytes [140]. Punt and coworkers have suggested a mechanism by which these auto reactive thymocytes are deleted in the thymic medulla: the simultaneous engagement of TCR and the molecule CD28 [141]. In the CD28-dependente mechanism, thymocytes recognizing self-antigens on thymic medullary epithelial cells expressing the stimulatory B7.1 molecule are killed by signals generated by simultaneous engagement of TCR (with pMHC) and CD28 (with B7.1). Gao and colleagues have also reported that the perinatal treatment with anti–B7-1 and anti–B7-2 prevents T cell clonal deletion *in vivo*, and leads to an accumulation of T cells capable of inducing T cell fatal multiorgan inflammation [142]. Also, programmed death-1 (PD-1), a member of the B7/CD28 family of costimulatory receptors, and its ligand, PD-1 ligand 1 (PD-L1), can also be directly implicated in thymocyte apoptosis since PD-1 ligation decreases phosphorylation of ERK and inhibits Bcl-2 up-regulation, both of which are critical for thymocyte maturation [143].

It has been also proposed that TCR-CD28 co-engagement may directly initiate an apoptotic program or may up-regulate a receptor specialized in the transduction of a death signal (the DRs). Indeed, death receptors such as tumor necrosis factor (TNF) receptor and DR3 have been implicated in thymocyte negative selection [144,145]. However, as a result of conflicting reports, it is not yet clear whether these DRs trigger a caspase cascade in thymocytes undergoing clonal deletion [146].

A large body of evidence has suggested that both JNK and p38 MAPK play critical role during the negative selection process of thymocytes since they are highly activated in response to intrathymic

signals in vivo [99,147-151]. p38 MAPK and JUNK are activated by upstream MAPK kinases (MAPKK) termed, respectively, MEK3/MEK6 and MEK4/MEK7, and their substrates include other kinases, cytosolic proteins and transcription factors through phosphorylation [152]. Once activated p38 and JUNK phosphorylate the pro-apoptotic molecule Bim, resulting in its translocation to mitochondria and an increase in apoptotic activity [153].

Currently, it is held that the pro-apoptotic molecule Bim and the nuclear orphan steroid receptor Nur77,a member of the Nurnuclear receptor family of intracellular transcription factors, play an especially important role in the death of thymocytes presenting high affinity TCR/pMHC interactions. Nur77 become activated through the ERK5 MAPK signaling cascade that acts by sequential activation of MEKK2/3, MEK5, ERK5 and myocyte enhancer factor 2 (MEF-2) [154]. Although Nur77 family consists of three members, Nur77, Nor-1, and Nurr1, only Nur77 and Nor-1 are induced in thymocytes in response to strong engagement of their TCR and correlates with apoptosis [155].

Two mechanisms of action, which are not mutually exclusive, have been proposed to Nur77: a transcription-dependent mechanism involving genes up regulation and a transcription-independent mechanism involving translocation to mitochondria, leading to cytochrome *c* release.

Rajpal et al., have shown that Nurr77 induces apoptosis in thymocytes through transcriptional activation of known pro-apoptotic genes, such as FasL and TRAIL, and also of the novel genes Nur77 Downstream Gene 1 and 2 (NDG1 and NDG2).Although the role of caspases in negative selection is controversial, these authors have shown that NDG1 encodes a novel protein that may initiate apoptosis through caspase-8 [156].

A growing body of evidence from recent studies, however, suggests that mitochondrial targeting of Nur77, but not its transcriptional activity, is essential for its pro-apoptotic effect. Accordingly, Nur77 translocates to mitochondria through interaction with Bcl-2, resulting in cytochrome *c* release and apoptosis by conversion of Bcl-2 from an anti- to pro-apoptotic mediator during negative selection [154,157]. These data might reconcile conflicting results found so far, showing that the defective negative selection in $Bim^{-/-}$ mice is only inefficiently blocked by overexpression of Bcl-2 [153,158]. Thompson and Winoto have suggested a new model where negative selection would work through two effector molecules that converge at the mitochondria via their interaction with Bcl-2 molecule: "while Bim antagonizes Bcl-2, Nur77 converts Bcl-2 to a killer form" [154].

It is also important to note that not all thymocytes expressing high avidity/affinity to self-peptides are excluded during the process of negative selection. Cells displaying regulatory functions, such as $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells (nTregs), NKT and CD8ααT cells, are also generated during thymocyte development by strong TCR/pMHC interactions [159]. However, exactly how these interactions occur to initiate different signals with distinct cellular consequences is not clear until now and so many unanswered questions remains to be clarified [98].It has been recently reported that transforming growth factor-beta (TGF-β) has a critical function for promoting nTreg cells survival during the negative selection process [160]. By using a model of TGF-β receptor-deficient cells nTreg, the authors have shown that apoptosis in such cells is associated with high expression of proapoptotic proteins Bim, Bax, and Bak and low expression of the antiapoptotic protein Bcl-2.

So, by the intrathymic mechanism of negative selection, autoreactive thymocytes are eliminated through apoptosis and only self-tolerant T cells are exported to the periphery.

The thymic microenvironment in infectious disease: an altered place for developing T cells

Literature has demonstrated that the thymus undergoes intense atrophy during viral and parasitic infectious diseases [reviewed in 161]. So, it is plausible to suppose that structural and morphological alterations in the thymic microenvironment, which are induced by direct or indirect effects of different pathogens, can impair positive and negative selection processes and lead to the entrance of immature potentially self-reactive or non-self-tolerant lymphocytes, or even both, into the peripheral circulation.

The deleterious effects on the thymus during infections can be reproduced in experimental models using either intact microorganisms or products such as cell wall components and toxins. The administration i*n vivo* of the bacterial superantigen staphylococcal enterotoxin B (SEB) produced by *Staphylococcus aureus*, for example, leads to thymus atrophy that is associated with thymocyte depletion [162]. The thymus is also a target organ in acute experimental Chagas' disease caused by the protozoan parasite *Trypanosoma cruzi*. This parasite causes alterations in the thymic microenvironment that include increased levels of apoptosis, particularly of cortical thymocytes bearing the phenotype $CD4^+CD8^+$, and an altered profile of intrathymic migratory responses of thymocytes that is correlated with the presence of potentially autoreactive thymus-derived immature DP cells in peripheral lymphoid organs of infected animals[163].

We have also studied thymic atrophy using different experimental models of infection. Thymic alterations in mice infected with *Paracoccidioides brasiliensis*, a dimorphic fungus that causes the most prevalent form of systemic mycosis in Brazil, include loss of corticomedullary delimitation, presence of a juxtacapsular inflammatory infiltrate and cortical degeneration caused by increased levels of apoptosis in DP thymocyte [164]. More recently, we have reported that the thymus gland is also a target organ during experimental infection with *Plasmodium berghei*, the causative agent of Malaria [165].The severe thymic atrophy observed during this infection is mainly characterized by increased depletion of intrathymic DP thymocytesand the presence of immature thymocytes (mainly DN and DP) in mesenteric lymph nodes and spleen [166].

Another aspect deserving attention is the fact that the thymus can be directly affected by pathogens, including viruses, parasites, and fungi, contradicting the idea that T cell maturation occurs at an antigen-free site [163-169]. The infection of thymic cells raises the hypothesis of the generation of central immunological tolerance for at least some antigens derived from the infectious agent. This issue still remains unexplored and represents a potentially important field of investigation.

Since T cell differentiation in the adult thymus depends on sequential interactions between lymphoid progenitors and stromal cells found in distinct regions of the cortex and medulla, it is most probably that thymic alterations observed during infectious diseases may result in a disruption of the normal intrathymic T cell development, which may lead to an altered exportation of T cell to the periphery, with severe consequences on the control of the immune response against the invading pathogen.

We suppose that more studies on the thymus under biological pressure of a given infection can contribute to better understand the behavior of this organ in respect to thymocyte development for the generation of an appropriate T cell repertoire.

Concluding Remarks

The survival of developing T cells is a complex and tightly regulated process that depends on signals the cells receive from the thymic microenvironment, like cytokines, chemokines, and mainly from the TCR/pMHC interaction.By preventing the maturation of thymocytes bearing TCR with no or insufficient affinity for self-MHC molecules, the positive selection process promotes the development of T cells with self MHC-restricted TCRs. By preventing the maturation of thymocytes bearing TCR with high affinity/avidity for self-peptides, the negative selection process ensures that thymocytes leaving the thymus are tolerant to the host's own proteins and thus contributes to prevention of autoimmunity.

It is noteworthy that during T cell development some $CD4^+$ SP thymocytes do not acquire the functional feature of helper cells, which trigger and/or enhance an immune response in the periphery, but rather differentiate into regulatory T cells, which block the immune response.

Studying the process of positive and negative selection of thymocytes is extremely difficult. Fetal thymus organ cultures are not well suited to distinguish between negative selection and a failure of positive selection. Also, the use of normal thymocytes is limited because they are fated to undergo apoptosis when placed in culture. On the other hand, the use of tumor cell lines has been hampered by the resistance that several of these lines display to death inducible by TCR engagement. The use of transgenic mice has increased dramatically in recent years and can contribute to our knowledge about T cell development. However, because transgenesis may alter a balanced genotype and produce unpredictable effects, careful interpretation of the results is advised. Another approach that can be explored is the study of the intracellular signals that discriminate between thymocyte-positive and -negative selection in an altered microenvironment induced by different pathogens.

Exploring the intrathymic events that are involved during T cell development provides an exciting research avenue since extremely important issues that need to be resolved in details for our complete understanding of the immune system still remain.

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