

The role of apoptosis in the development and function of T lymphocytes

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ABSTRACT

Apoptosis plays an essential role in T cell biology. Thymocytes expressing nonfunctional or autoreactive TCRs are eliminated by apoptosis during development. Apoptosis also leads to the deletion of expanded effector T cells during immune responses. The dysregulation of apoptosis in the immune system results in autoimmunity, tumorigenesis and immunodeficiency. Two major pathways lead to apoptosis: the intrinsic cell death pathway controlled by Bcl-2 family members and the extrinsic cell death pathway controlled by death receptor signaling. These two pathways work together to regulate T lymphocyte development and function.

Keywords: thymocyte, effector, memory T lymphocytes, apoptosis.

INTRODUCTION

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of apoptosis leads to a variety of immune disorders, including both immunodeficiency and autoimmunity. In this review, we will address three topics: i) apoptosis pathways; ii) T lymphocyte development and apoptosis; and iii) T lymphocyte function and apoptosis.

APOPTOSIS PATHWAYS

Apoptosis, a genetically conserved pathway in all metazoans, is both essential for the generation of multicellular tissues during embryonic development as well as the maintenance of cellular homeostasis [1]. The typical morphological changes during apoptosis include membrane blebbing, DNA fragmentation and cellular component degradation. Notably, these changes occur without the loss of membrane integrity. During the past two decades, extensive work has been done to elucidate the molecular mechanism of apoptosis. From this large body of work it is clear that apoptosis is induced by a range of stimuli that activate two major cell death signaling pathways: the intrinsic pathway mainly controlled by Bcl-2 protein

family members and the extrinsic pathway activated by death receptors of the tumor necrosis factor receptor superfamily (Fig. 1).

The intrinsic cell death pathway

The intrinsic cell death pathway is activated by a variety of apoptotic stimuli, such as genomic toxicity and cytokine withdrawal. Intrinsic cell death signals generally converge within the cell at the outer membrane of mitochondria. They result in a loss of mitochondrial membrane integrity and the subsequent activation of downstream apoptotic pathways.

Bcl-2 family members control mitochondrial membrane integrity and are the major mediators in the intrinsic cell death pathway (Fig. 1). The founding member of this family, Bcl-2, was first identified at the interchromosomal breakpoint of the t(14;18) translocation in follicular B cell lymphoma [2]. Other family members were discovered based on sequence homology in four α -helical segments named Bcl-2 Homologous Domains (BH1-4) [3-5]. Bcl-2 protein family members are divided into three subfamilies based on function and BH domain structure. These groupings include the anti-apoptotic BH1-4 subfamily, the pro-apoptotic BH1-3 subfamily and the pro-apoptotic BH3-only subfamily.

ANTI-APOPTOTIC BH1-4 SUBFAMILY

The members of the BH1-4 subfamily include Bcl-2, Bcl-x_L, Mcl-1 and A1/Bfl-1 which all contain four BH domains. Members in this subfamily are anti-apoptotic in

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most experimental systems. They contain a transmembrane domain at the C-terminus and mainly localize to the outer membrane of the mitochondria where they inhibit the function of pro-apoptotic Bcl-2 family members via BH domain interactions. Bcl-2, Bcl-x_L, Mcl-1 and A1 are all actively involved in T lymphocyte development and survival.

Bcl-2 protects various cell types from apoptosis. During T cell development, the expression for Bcl-2 is tightly regulated, which suggests a critical role of Bcl-2 in T lymphocytes. Indeed, Bcl-2^{-/-} mice exhibit an intrinsic defect in T lymphocyte development probably due to increased apoptosis [6, 7]. In Bcl-2^{-/-} adult bone marrow hematopoietic stem cell reconstituted chimeric mice, donor-derived lymphocyte development is almost completely absent [8]. IL-7 provides a crucial survival signal for lymphocyte precursors. It is believed that Bcl-2 plays an important anti-apoptotic role downstream of IL-7 signaling [9, 10].

A large body of work has suggested a critical role for Bcl-x in protection of different cell types from apoptosis. Five isoforms of Bcl-x (Bcl-x_L, Bcl-x_S, Bcl-x_γ, Bcl-x_β and Bcl-x_{ΔTM}) generated by alternative splicing of mRNA have been identified [11-15]. Bcl-x^{-/-} mice die by embryonic day 13 due to extensive cell death in the central nerve system where Bcl-x_L is highly expressed [16]. Furthermore, the deficiency of Bcl-x in erythroid cells causes hemolytic anemia and profound splenomegaly [17]. Recently, it has been demonstrated that Bcl-x is required for dendritic cell survival [18, 19]. Therefore, Bcl-x_L protects different cells from apoptosis *in vivo*.

Bcl-x_L is the major isoform in T lymphocytes. During T lymphocyte development, the expression of Bcl-x_L is tightly regulated [20, 21]. Bcl-x_L is not expressed at DN or SP stage. However, it is highly expressed in DP thymocytes. The high level Bcl-x_L expression at DP stage is required for the survival of DP thymocytes. DP thymocytes from Bcl-x^{-/-}Rag2^{-/-} chimeric mice [16, 21] and RORγ^{-/-} mice, which have a profound reduction of Bcl-x_L expression at the DP stage [22, 23], have a reduced lifespan. Furthermore, this defect in RORγ^{-/-} mice can be rescued by a Bcl-x_L transgene. These data demonstrated that Bcl-x_L plays a non-redundant role to promote DP thymocyte survival.

Recently, Mcl-1 has also been shown to play an essential role in lymphocyte survival [24]. Mcl-1 is a short-lived protein with a very fast turn-over rate. It is thought that Mcl-1 functions as a sensitive switch to decide cell fate in response to survival signals. Early deletion of Mcl-1 during lymphocyte development causes a blockade at the pro-B stage for B lineage cells and a blockade at the DN stage for T lineage cells. Moreover, the deletion of

Mcl-1 in mature lymphocytes also leads to apoptosis. IL-7 signaling significantly upregulates Mcl-1 expression. Based on these data, it has been suggested that Mcl-1 is a critical pro-survival molecule downstream of IL-7 signaling [24]. In addition, hematopoietic stem cells (HSCs) require Mcl-1 for their survival, and stem cell factor (SCF) signal greatly enhances Mcl-1 expression in mouse HSCs [25]. Therefore, Mcl-1 is an essential anti-apoptotic protein both in lymphocytes and HSCs.

A1/Bfl-1 is a fourth member of the anti-apoptotic Bcl-2 subfamily. A1 prevents cell death as a downstream effector of NF-κB signaling [26-29]. A1/Bfl-1 has been identified as a transcriptional target of pre-TCR signal. The knockdown of endogenous A1/Bfl-1 expression by siRNA impairs pre-TCR⁺ pre-T cell line survival in response to pre-TCR signaling [30]. Expression of A1/Bfl-1 rescues DN3 thymocytes from apoptosis by inhibiting the function of caspase 3 indirectly [30]. Therefore, A1 plays an anti-apoptotic role in response to pre-TCR signaling during T cell development.

In summary, anti-apoptotic Bcl-2 family members play critical roles at different stages during T lymphocyte development.

PRO-APOPTOTIC BH1-3 SUBFAMILY

The pro-apoptotic BH1-3 subfamily includes the pro-apoptotic proteins Bax, Bak and Bok, which contain three BH domains (BH1-3). The expression of Bok is restricted to the reproductive system, whereas Bax and Bak have a broad expression pattern [31-35]. Based on work done on Bax^{-/-}Bak^{-/-} double deficient mice, it is believed that Bax and Bak are the main cell death executioners in the intrinsic cell death pathway [36-38]. Bax^{-/-}Bak^{-/-} cells are highly resistant to a variety of the intrinsic cell death pathway stimuli including growth factor withdrawal and DNA damage. In addition, initiator caspase activation is abolished in Bax^{-/-}Bak^{-/-} cells [39]. It is generally thought that Bax and Bak perform their function by forming super-molecular openings in the outer membranes of mitochondria. This is supported by the fact that Bax, pro-apoptotic BH3-only protein Bid or Bid BH3 peptide and the unique mitochondrial membrane component cardiolipin form a super-molecular 'hole' in purified mitochondrial outer membranes in a cell free system. Bcl-x_L inhibits the formation of the super-molecular holes by Bax, Bid and cardiolipin [40]. Super-molecular openings in the outer membrane of mitochondria leads to the loss of mitochondrial integrity and the release of pro-apoptotic factors including cytochrome C, SMAC/Diablo, Omi/HtrA2, DNase endonuclease G and apoptosis inducing factor (AIF) [1].

These pro-apoptotic molecules activate downstream apoptotic machinery. Once cytochrome C enters the

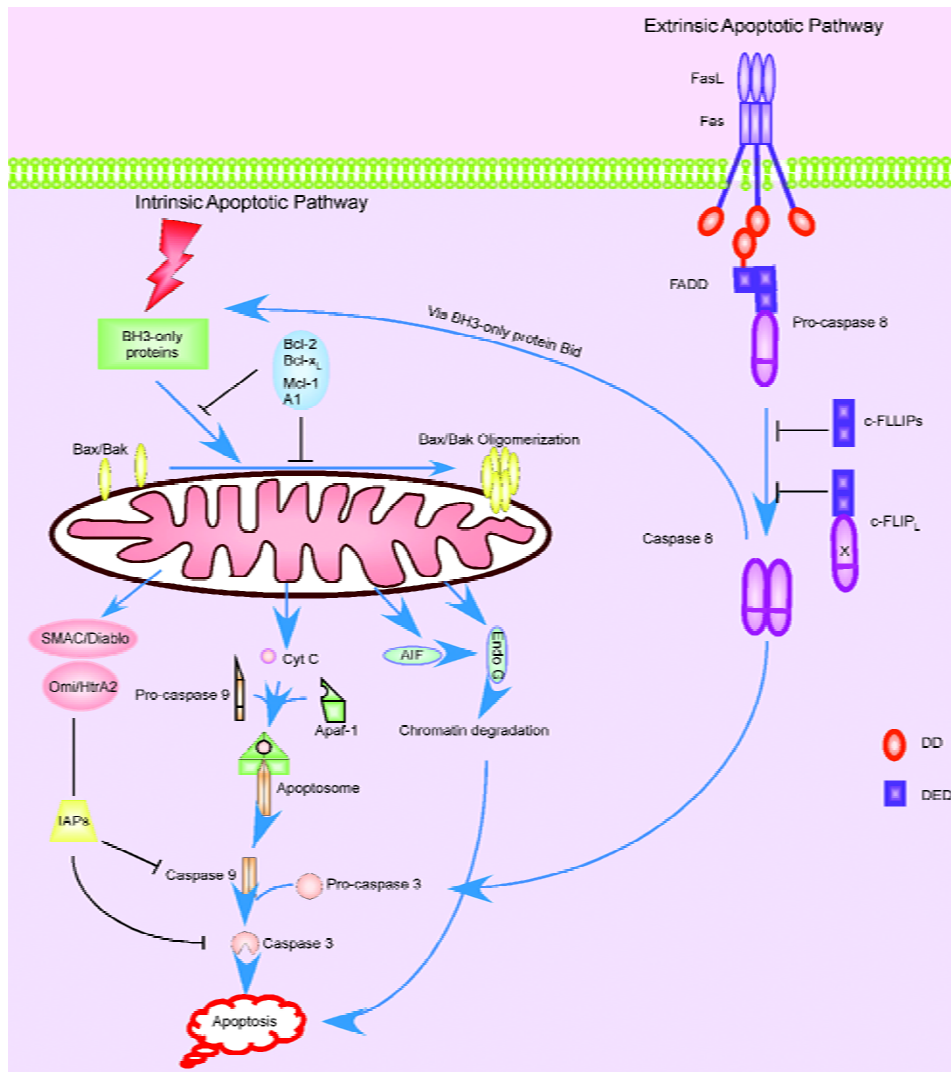


Fig. 1 Apoptotic pathways. Two major pathways lead to apoptosis: the intrinsic cell death pathway controlled by Bcl-2 family members and the extrinsic cell death pathway controlled by death receptor signaling.

cytoplasm, it binds to Apaf-1 and pro-caspase 9. Cytochrome C, Apaf-1 and pro-caspase 9 then form a supermolecular complex called the apoptosome with the help of dATP. The formation of the apoptosome leads to the cleavage of pro-caspase 9 and the generation of active caspase 9 [41-43]. Active caspase 9 further activates downstream effector caspases, including caspase 3, 6 and 7, which in turn cleave hundreds of cellular components and result in irreversible cell death.

Caspases are a family of cytosolic cysteine proteinases that cleave their substrates after an aspartic acid residue [44]. Caspases are actively involved in inflammation and apoptosis. Caspase 2, 3, 6, 7, 8, 9 and 10 are the major caspases involved in apoptosis. Most other caspases are

involved in inflammation. Caspases 2, 8, 9 and 10 are initiator caspases, and caspase 3, 6 and 7 are effector caspases. Caspases are initially synthesized as an inactive zymogen, which contains an N-terminal pro-domain. For example, pro-caspase 8 contains two DED (death effector domain) domains as the pro-domain and pro-caspase 9 contains one CARD (caspase recruitment domain) domain as the pro-domain. In all pro-caspases, besides the N-terminal pro-domain, there is also one large domain of 20 kDa and one small domain of 10 kDa at the C-terminus. Cleaving the small domain and the large domain separately from the pro-domain leads to caspase activation. Caspases are the major mediators downstream of both intrinsic and extrinsic apoptotic pathways.

IAPs (inhibitor of apoptosis proteins) are a group of cytoplasmic proteins which are potent inhibitors of caspases [45-49]. IAPs add another layer of regulation to intrinsic apoptotic pathways to ensure that unnecessary caspase activation does not occur. IAPs are regulated by two proteins released from mitochondria. One is SMAC/Diablo, the other is Omi/HtrA2. SMAC/Diablo specifically binds to IAPs and releases IAP-mediated caspase inhibition. Specific binding of SMAC/Diablo to IAPs depends on four amino acid residues at the N-terminus of SMAC/Diablo [50-53]. Omi/HtrA2 has a similar IAP inhibitory function as SMAC/Diablo. However, the pro-apoptotic function of Omi/HtrA2 requires its serine proteinase activity [54]. Therefore, SMAC/Diablo and Omi/HtrA2 promote caspase activation by the removal of IAP inhibition.

Caspase independent mechanisms are also involved in the intrinsic death pathway downstream of Bax and Bak. Two proteins, DNase endonuclease G and AIF, mainly activate caspase independent pathways leading to apoptosis. Once released from the mitochondria, DNase endonuclease G translocates into the nucleus to initiate internucleosomal chromatin degradation and apoptosis [55, 56]. AIF is also a mitochondrial protein. Once released into the cytosol, AIF translocates into the nucleus and induces chromatin condensation and large scale chromatin degradation. AIF-induced apoptosis is caspase-independent and poly (ADP-ribose) polymerase-1-dependent. Furthermore, AIF may coordinate with endonuclease G to degrade chromatin [57-60].

In summary, Bax and Bak control the integrity of mitochondria and the release of many pro-apoptotic molecules from the mitochondria. Once released from the mitochondria, the pro-apoptotic molecules activate cell death via a caspase-dependent or a caspase-independent mechanism.

PRO-APOPTOTIC BH3-ONLY SUBFAMILY

Most members in the BH3-only subfamily only contain one BH3 domain. All members are pro-apoptotic by either promoting the function of Bax and Bak or inhibiting the function of anti-apoptotic Bcl-2 family members [61, 62]. BH3-only proteins include Bad, Bid, Bik, Bim, Noxa and Puma. Different BH3-only proteins have different binding affinities for different anti-apoptotic Bcl-2 family members. Bim and Puma tightly bind to all anti-apoptotic Bcl-2 family members, while Bad only binds to Bcl-2 and Bcl-x_L. Bik only binds to Bcl-x_L and A1, and Noxa only binds to Mcl-1 and A1 [63]. BH3-only proteins function as sentinels instead of direct executioners of cell death. Different cell death stimuli lead to the activation of different BH3-only proteins. Bid is activated by caspase 8 in response to death receptor signaling [64-66]. Bad senses growth fac-

tor withdrawal and translocates from the cytosol to the mitochondrial outer membrane [67, 68]. Bim is downstream of TCR signal induced apoptosis as Bim^{-/-} mice have a dramatic defect in clearance of T lymphocytes after TCR stimulation [69]. Noxa and Puma is mainly responsible for DNA damage induced apoptosis [70].

Bid functions as a linker between the extrinsic and intrinsic cell death pathways [64-66]. Bid senses extrinsic death signals from death receptors. After death receptor activation, caspase 8 is activated. Active caspase 8 cleaves Bid and generates a smaller fragment called tBid. tBid translocates to the mitochondrial outer membrane and inhibits the function of the anti-apoptotic protein Bcl-2 and Bcl-x_L, which leads to the activation of the intrinsic death pathway. Bid deficient mice are highly resistant to Fas-induced liver injury [71]. Therefore, Bid functions as a critical component to amplify extrinsic death signal through intrinsic death pathway.

Bad is a sensor for growth factor withdrawal as growth factor signals hold the BH3-only protein Bad in an inactive form. Growth factor signals activate Akt kinase, which phosphorylates Bad. Phosphorylated Bad is sequestered in the cytosol by the adaptor protein 14-3-3. After growth factor withdrawal, dephosphorylated Bad translocates onto the outer membrane of mitochondria and leads to apoptosis [67, 68].

Puma and Noxa are involved in DNA damage induced cell death. The expression of Puma and Noxa is highly induced in response to irradiation in a p53-dependent manner [72-74]. The deletion of Puma in human colon cancer cell lines or the knockdown of Noxa expression in MEFs (mouse embryonic fibroblasts) abolishes p53-dependent apoptosis [75, 76]. The studies on Noxa^{-/-} and Puma^{-/-} mice have shown that Noxa deficiency partially rescues MEFs from DNA damage induced apoptosis. Puma deficiency confers thymocytes, lymphocytes and fibroblasts resistance to p53-dependent apoptotic stimuli. Interestingly, Puma^{-/-} cells are also resistant to some p53-independent death signals, such as cytokine withdrawal, dexamethasone and phorbol ester 12-myristate 13-acetate (PMA) treatment [70]. These data suggest that Noxa and Puma are the main mediators of DNA damage induced apoptosis and Puma may have a broader function than Noxa.

Another member of this subfamily, which may be related to the function of immune system, is Bik. Bik is broadly expressed in the hematopoietic system. However, Bik^{-/-} mice do not have any significant defects in apoptosis [77], suggesting that there is a redundant or non-essential role for Bik in the immune system.

In general, different cell death stimuli activate or induce expression of different BH-3 only proteins. At the outer membrane of mitochondria, BH-3 only proteins titrate out

the anti-apoptotic effect of pro-survival Bcl-2 family members. Therefore, when Bax and Bak are activated, mitochondrial integrity is disrupted and pro-apoptotic factors held inside the mitochondria are released into the cytoplasm. Apoptosis proceeds mainly through activation of downstream caspases.

The extrinsic cell death pathway

The tumor necrosis factor (TNF) superfamily and TNF receptor superfamily are evolutionarily conserved protein families involved in various biological processes [78]. At least 19 members of the TNF superfamily and 32 members of the TNFR superfamily have been identified in the human genome [79]. Members of the TNF superfamily and TNFR superfamily are broadly expressed in the immune system and actively involved in inflammation, adaptive immunity, lymphoid organogenesis and immune homeostasis [78]. Many members of the TNF and TNFR superfamilies have been chosen as therapeutic targets or potential targets for the treatment of a variety of different human diseases, such as autoimmunity, osteoporosis and cancer.

Based on sequence homology and cellular function, the TNF receptor superfamily is classified into three groups: the first group are death receptors, which contain a death domain (DD) in their cytoplasmic tail; the second group do not contain a DD in their cytoplasmic tail, instead, they contain one or more TNF receptor associated factor (TRAF)-interacting motifs; the third group are decoy receptors which do not contain any signaling motifs in their cytoplasmic tails. Decoy receptors competitively bind to ligand and inhibit the function of the other TNFR superfamily members [78]. Death receptors are initiators of the extrinsic death pathway (Fig. 1).

Death receptors (Group 1) include Fas, TNFR1, death receptor 3 (DR3), tumor necrosis factor apoptosis related ligand (TRAIL) receptor1, TRAIL-R2 and DR6 [80]. Death receptors all contain a DD in their cytoplasmic tail [81]. The death domain is an evolutionarily conserved domain composed of approximately 60 amino acids that forms a globular bundle of six α helices. After ligands bind to death receptors, the DD mediates homotypic interaction with other DD-containing adaptor proteins with high specificity. There are two major DD-containing adaptor proteins involved in death receptor signaling: Fas associated death domain (FADD) and TNF receptor associated death domain (TRADD) [82, 83]. FADD binds to Fas, TRAIL-R1 and TRAIL-R2, while TRADD preferentially binds to TNFR1, DR3 and DR6 [82-87]. The signal pathways for Fas and TNFR1 have been well established.

FAS MEDIATED EXTRINSIC CELL DEATH PATHWAY

Fas and FasL have been extensively studied in the im-

mune system. It is well-known that Fas-FasL interactions play an essential role in maintaining homeostasis of the immune system. Both humans and mice carrying a mutated Fas develop a deleterious lymphoproliferative and autoimmune disease [88, 89]. *In vitro* activation induced cell death (AICD) in T lymphocytes is mainly mediated by Fas-FasL interactions [90]. Furthermore, Fas mediated killing is one of the important ways employed by cytotoxic T cells [91]. Therefore, Fas delivers a critical death signal in the immune system.

Activation of Fas delivers an apoptotic signal through FADD and caspase 8/10. After FasL binds to Fas, the DD domain in the cytoplasmic tail of Fas recruits the adaptor protein FADD. FADD is a 26 kDa cytosolic adaptor protein composed of two functional domains: an N-terminal death effector domain (DED) and a C-terminal death domain (DD). The DED and the DD domain have homology at the amino acid level. Like the DD domain, the DED domain also mediates homotypic interaction between proteins containing DED domains. After the DD domain of FADD binds to Fas, the DED domain of FADD recruits pro-caspase 8 and also pro-caspase 10 in human cells, which leads to the activation of caspase 8/10. Caspase 10 is highly homologous to caspase 8 at both a sequence level and a function level. However, there is no murine homolog for caspase 10 [92-94].

Active caspase 8 activates a downstream caspase cascade and leads to cell death. Because most receptors in the TNFR superfamily exist as a homotrimer on cell surface, pro-caspase 8 molecules are brought in close proximity to each other after binding to FADD, leading to auto-activation of pro-caspase 8 [95, 96]. Active caspase 8 cleaves and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis. Furthermore, caspase 8 cleaves the BH3-only protein Bid and generates a small fragment of Bid, tBid. tBid translocates to the mitochondria, inhibits the effect of anti-apoptotic Bcl-2 family members, and induces apoptosis through the intrinsic death pathway [65, 66].

c-FLIP is a critical negative regulator of the extrinsic cell death pathway. c-FLIP (also called CASH, Casper, CLARP, FLAME-1, I-FLICE and usurpin) is a homolog of pro-caspase 8. It does not have caspase activity and functions as a competitive inhibitor of caspase 8 [97-104]. In the mouse genome, the c-FLIP gene is only 40 kb away from caspase 8. In the human genome, the c-FLIP gene is 80 kb away from caspase 8 while caspase 10 is positioned between c-FLIP and caspase 8. This genomic structure suggests a recent duplication event. Two isoforms of c-FLIP, generated by mRNA alternative splicing, have been confirmed at the protein level: 55 kDa c-FLIP_L and 26 kDa c-FLIP_S. Like pro-caspase 8, c-FLIP_L has two DED do-

mains at the N-terminus and a caspase-like domain at the C-terminus (Fig. 1). However, c-FLIP_L does not have enzymatic activity due to the absence of several critical amino acids, including a cysteine residue within the QACXG motif and histidine residue within the HG motif [105]. Both motifs are highly conserved in all caspases and essential for caspase activity. c-FLIP_S contains two DED domains without any caspase-like domains [105]. Both c-FLIP_L and c-FLIP_S are efficiently recruited to the death inducing signaling complex (DISC) upon death receptor stimulation and inhibit pro-caspase 8 activation at the DISC. However, when expressed at a low level, c-FLIP_L has been shown to promote pro-caspase 8 processing [106]. In the genome of some viruses, there are FLIP like genes called v-FLIP. v-FLIP genes encoded in the γ -herpesvirus genome have a similar domain structure as c-FLIP_S. Like c-FLIP, v-FLIP is recruited to the DISC and inhibits apoptosis in infected cells, which may provide a protective mechanism against Fas-mediated killing [105]. In general, it is believed that c-FLIP inhibits death receptor signaling and caspase 8 activation.

c-FLIP deficient mice die early during embryonic development due to impaired heart development. Experiments performed on c-FLIP^{-/-} mouse embryonic fibroblast (MEF) cells have confirmed a critical role of c-FLIP in protection against death receptor induced apoptosis *in vivo* [107].

TNFR1 MEDIATED EXTRINSIC CELL DEATH PATHWAY

In most cells under normal conditions, TNFR1 signaling induces an inflammatory response through NF- κ B. However, when protein synthesis or NF- κ B signaling is inhibited, TNFR1 ligation becomes potentially apoptotic [80].

After TNF α binding, the adaptor protein TRADD is recruited to TNFR1. TRADD is a 34 kDa cytosolic adaptor protein containing one DD domain. After TRADD binds to the DD domain of TNFR1, a signaling complex forms on the cytoplasmic tail of TNFR1 [83]. This signaling complex is composed of TNFR1, TRADD, TNF receptor associated factor 1 (TRAF1), TRAF2 and receptor interacting protein 1 (RIP1). RIP1 is a DD domain-containing protein kinase that promotes NF- κ B activation and inflammatory responses [108, 109]. The main function of this TRADD, TRAF1/2 and RIP1 complex is to activate NF- κ B and JNK pathways [110, 111]. NF- κ B is the major pro-inflammatory and pro-survival transcription factor in mammalian cells. NF- κ B activates the transcription of c-FLIP, anti-apoptotic Bcl-2 family members and IAPs [26, 112, 113]. Meanwhile, besides pro-inflammatory and pro-survival NF- κ B activation complex, FADD and caspase 8/10 are also recruited to TRADD. FADD and caspase 8/10 form a signaling complex, activate caspase 8/10 and in-

duce apoptosis [110, 111]. How one receptor can activate two distinct signaling pathways that lead to opposite outcomes has been a puzzling question for years.

Recently, it has been shown that TNFR1 signaling leads to the sequential formation of two distinct signaling complexes in cell lines [114]. After TNF α binds to TNFR1, signaling complex I forms immediately on the cytoplasmic tail of TNFR1. Signaling complex I includes TNFR1, TRADD, TRAF2, RIP1 and cIAP1. NF- κ B pathways are activated by signaling complex I induced signal. Therefore, signaling complex I only delivers a pro-inflammatory signal. When signaling complex I forms on TNFR1, mono-ubiquitination or poly-ubiquitination of RIP1 and TRADD occur [115]. It is speculated that these post-transcriptional modifications may cause components of signaling complex I to leave TNFR1 and the cell membrane [116].

Several hours after TNF α signaling, signaling complex II forms in the cytoplasm. Signaling complex II is composed of TRADD, RIP1, FADD and caspase 8/10 without TNFR1. This model explains the previous observation that although TNFR1 activates caspase 8, no direct interaction is detected between TNFR1, FADD and caspase 8 [117]. Apoptotic signaling is delivered by signaling complex II. It seems paradoxical that RIP1, which mainly functions as an activator of pro-inflammatory NF- κ B pathway, is included in the pro-apoptotic signal complex II. However, it has been shown that RIP1 is a potential substrate for caspase 8 [118]. After being cleaved by caspase 8, the DD domain of RIP1 is released as a fragment called RIPc. RIPc is a dominant negative inhibitor of full-length RIP1 induced NF- κ B activation [118].

Although signaling complex II is a potent apoptosis activator, the outcome of signaling complex II depends on the function of signaling complex I. If signaling complex I can successfully activate NF- κ B, this transcription factor quickly induces the expression of several anti-apoptotic proteins such as c-FLIP before the formation of signaling complex II. Once the expression level of c-FLIP is high enough, c-FLIP efficiently incorporates into signaling complex II and inhibits the activation of pro-caspase 8/10. Therefore, apoptosis is inhibited. However, if NF- κ B cannot be efficiently activated and the expression level of c-FLIP is not high enough to inhibit the activation of pro-caspase 8/10, apoptotic signaling complex II activates cell death pathway [114].

This two-step separated signaling complex model helps elucidate the long-standing puzzle how TNFR1 signaling can lead to both cell survival and cell death. However, this simplified model cannot incorporate all the phenomena attributed to TNFR1 signaling. A recent paper has suggested an interesting function of JNK during TNFR1 induced apoptosis [119]. Transiently activated JNK is correlated

with survival while sustained JNK activation leads to apoptosis. Under normal condition, JNK is transiently activated by TNFR1 signaling through TRAF2. However, the blockade of NF- κ B leads to the sustained activation of JNK upon TNFR1 signaling. TNF α signaling induces the production of reactive oxygen species (ROS), which inhibits JNK-inactivating phosphatase. TNF α mediated NF- κ B activation induces the antioxidant enzyme superoxide dismutase, which inhibits the accumulation of ROS. Therefore, TNF α induced NF- κ B activation limits the sustained activation of JNK [120]. Sustained JNK activation causes the cleavage of BH3-only protein Bid and generation of a smaller fragment called jBid. jBid translocates onto the outer membrane of mitochondria and specifically releases SMAC/Diablo from the mitochondria, which results in the promotion of TNF α induced apoptosis [119]. Furthermore, JNK1^{-/-} or JNK2^{-/-} thymocytes are resistant to TCR/CD3 induced apoptosis, suggesting that JNK may deliver pro-apoptotic signal *in vivo* under certain circumstances [121].

Interestingly, another model, which is different, but not mutually exclusive from the above model, has been proposed for TNFR1 signaling [122]. Based on a new technique called ligand-specific magnetic labeling and purification of TNFR1 endocytic vesicles, this model proposes that TNFR1 endocytosis is essential for TNF α induced apoptotic signaling, but not for TNF α induced NF- κ B

activation. After TNF α signaling, RIP1 and TRAF2 bind to TNFR1 and form a signal complex for NF- κ B activation at the plasma membrane. Simultaneously, TNFR1 undergoes endocytosis after ligand binding. TRADD, FADD and pro-caspase 8 only bind to the endocytosed TNFR1 and form a death inducing signal complex (DISC) away from the plasma membrane to deliver a death signal. A TNFR1 internalization domain (TRID) has been identified. A point mutation in TRID abolishes the ability of TNFR1 to undergo endocytosis and to induce apoptosis, but still retains the ability to activate NF- κ B.

In summary, a tremendous amount of work has been done to illustrate the molecular mechanism of the intrinsic and extrinsic apoptotic pathways. It is generally believed that both intrinsic and extrinsic cell death pathways are critically involved in the development and function of T lymphocytes.

T LYMPHOCYTE DEVELOPMENT AND APOPTOSIS

T lymphocyte development is a highly regulated process. To ensure the generation of a functional TCR repertoire, there are multiple checkpoints during thymocyte development to delete thymocytes expressing a nonfunctional or autoreactive TCR. In the thymus, T lymphocytes are divided into three major developmental stages: the CD4⁺CD8⁻ double negative (DN) stage; the CD4⁺CD8⁺ double posi-

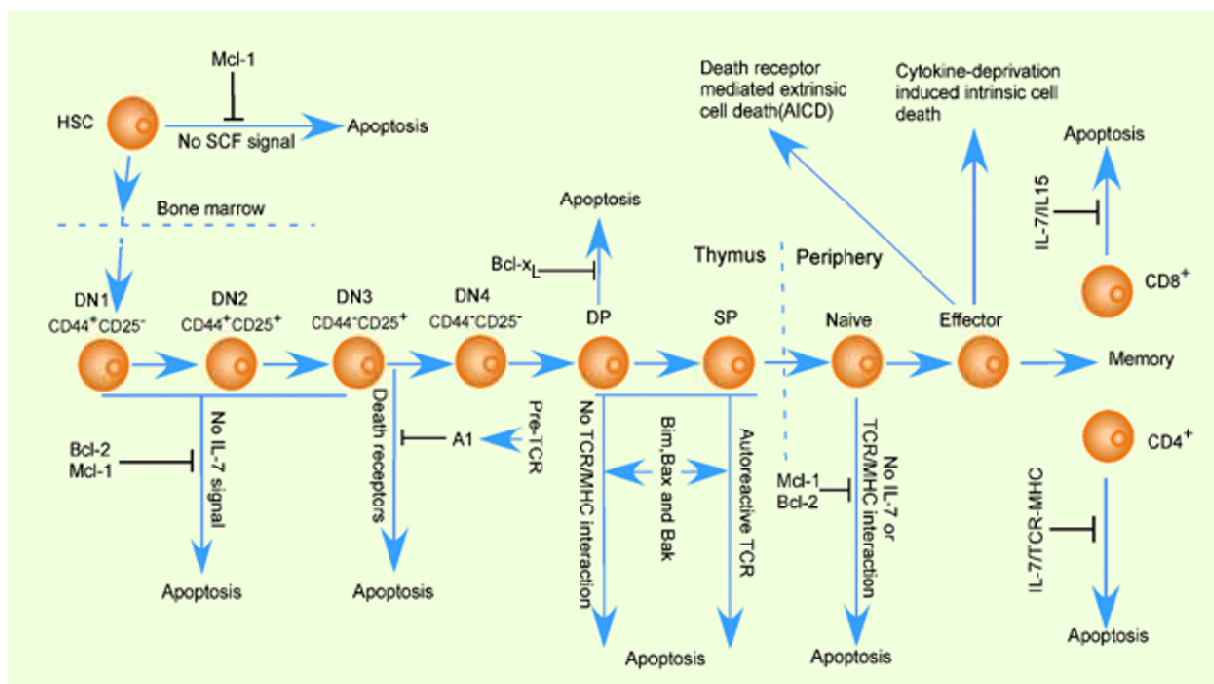


Fig. 2 The role of apoptosis in the development and function of T lymphocytes. Major pro-apoptotic and anti-apoptotic signals/molecules are shown.

tive (DP) stage and the CD4⁺CD8⁻ or CD8⁺CD4⁻ single positive (SP) stage [123-125]. At each stage, the major signals that trigger thymocyte apoptosis are different (Fig. 2).

DN thymocytes, IL-7 signaling and β selection

At the DN stage, T cell precursors undergo TCR β chain rearrangement. DN thymocytes are divided into four developmental stages DN1-DN4 by their surface expression of CD25 and CD44 (Fig 2). In adults, thymocyte development begins with the migration of multipotent precursors from the bone marrow into the thymus. These precursor cells periodically enter the thymus through post-capillary venules in the cortex region close to the corticomedullary junction [126, 127]. After entering the thymus, the precursor cells differentiate into DN1 cells (CD44⁺CD25⁻). As DN1 cells migrate towards the outer cortex, they progressively differentiate into DN2 (CD44⁺CD25⁺) and then DN3 (CD44⁻CD25⁺) cells. Chemokine receptor CCR7 signaling is critical for the migration of DN cells towards the outer cortex as DN2 cells accumulate at the corticomedullary junction in CCR7 deficient mice [128, 129]. Starting from DN2 and DN3, RAG1 and RAG2 genes are expressed and the TCR β locus undergoes vigorous genomic rearrangement. After TCR β rearrangement, thymocytes undergo β -selection to ensure that only cells with a successfully rearranged TCR β gene survive. These cells receive positive signals from the pre-TCR, which is composed of a TCR β chain and an invariant pre-T α chain. Successful β -selection leads to a series of events including proliferation and differentiation. Thymocytes that do not receive a β selection signal undergo apoptosis. After β selection, thymocytes further differentiate into the DN4 (CD44⁻CD25⁻) and immature single positive (ISP) (CD3⁺CD4⁺CD8⁺) cells.

During DN thymocyte development, the cytokine IL-7 has been shown to be a crucial pro-survival molecule as IL-7^{-/-} or IL-7R^{-/-} mice have fewer DN cells [130, 131]. The intrinsic pathway anti-apoptotic Bcl-2 family members are the main effector molecules downstream of IL-7 signaling. Bcl-2 is highly expressed at the DN stage and overexpression of Bcl-2 rescues T cell development in the absence of IL-7 signal [9, 10]. Recently, another anti-apoptotic Bcl-2 protein family member, Mcl-1, has been shown to be an essential pro-survival factor regulated by IL-7 signaling [24]. Loss of Mcl-1 expression leads to a development blockade at the DN stage due to increased apoptosis. Furthermore, IL-7 signal induces Mcl-1 expression [24]. Therefore, Bcl-2 and Mcl-1 are downstream of IL-7 signal and promote DN thymocyte survival.

β selection is a critical checkpoint at the DN3 stage mediated by pre-TCR signaling. Without a proper pre-TCR

signal, DN cells undergo apoptosis, which maybe mediated by both intrinsic and extrinsic apoptotic pathways. The anti-apoptotic Bcl-2 family member A1/Bfl-1 lies downstream of the pre-TCR and protects DN thymocytes from apoptosis in response to pre-TCR signaling [30]. The extrinsic cell death pathway may mediate apoptosis in DN3 cells that do not receive pre-TCR signals. It has been shown that death receptor 3 (DR3), death receptor 5 (DR5) and tumor necrosis factor receptor (TNFR)1 are expressed in DN3 [132]. Fas associated death domain adaptor (FADD) is an essential adaptor downstream of these death receptors. Transgenic expression of a dominant negative form of FADD (FADD-DN) rescues DN thymocytes which fail β -selection due to the inability to undergo gene rearrangement [132]. This data suggests that inhibition of death receptor signaling bypasses the requirement of pre-TCR signaling to promote DN thymocyte survival.

In summary, both intrinsic and extrinsic apoptotic pathways are actively involved in IL-7 and pre-TCR mediated thymocyte survival at DN stage.

DP thymocytes and death by neglect

DP thymocytes rearrange their TCR α chain and are subjected to positive and negative selection. The majority of DP thymocytes cannot generate a functional TCR and undergo death by neglect. Activation of the intrinsic death pathway may be the major mechanism underlying death by neglect.

At the DP stage, RAG-1 and RAG-2 expression is reactivated and the TCR α loci undergo rearrangement [123, 124]. Once a DP thymocyte expresses a successfully rearranged TCR molecule on its surface, it scans an array of self MHC-peptide complexes presented in the thymus. Only DP thymocytes expressing a TCR with intermediate affinity for self MHC-peptide complexes are positively selected [123, 124]. Thymocytes expressing a low affinity TCR undergo death by neglect to ensure a functional TCR repertoire in the periphery [123, 124]. Thymocytes expressing a high affinity TCR are subjected to negative selection to eliminate potentially autoreactive T cells [123, 124].

Death by neglect contributes to the majority of cell death during thymocyte development. It is estimated that 90% of developing thymocytes undergo death by neglect. The detailed molecular mechanism underlying death by neglect remains largely elusive. However, it is generally believed that Bcl-2 family members are the main effectors involved in death by neglect. In agreement with this notion, spontaneous thymocyte apoptosis *in vitro*, which may mimic *in vivo* death by neglect, is controlled by the intrinsic cell death pathway with little or no death receptor signaling involvement. Degradation of the anti-apoptotic proteins Bcl-2 and Bcl-x_L and the release of cytochrome C into the cyto-

plasm are the early events of spontaneous thymocyte apoptosis. Accordingly, the pan-caspase inhibitor zVAD inhibits spontaneous thymocyte apoptosis *in vitro* [133].

$Bcl-x_L$ is highly expressed in DP thymocytes and promotes the life-span of DP thymocytes. In $Bcl-x^{-/-}$ $Rag^{-/-}$ chimeric mice, DP thymocytes have a decreased life-span *in vitro* [16]. $ROR\gamma^{-/-}$ mice, which have dramatically decreased $Bcl-x_L$ expression in DP thymocytes, display both a decreased life-span for DP thymocytes as well as a defect in TCR α gene segments usage. These defects are rescued by a $Bcl-x_L$ transgene [22, 23]. The BH3-only protein Bim is also involved in spontaneous thymocyte apoptosis [134]. During a three-day *in vitro* culture, sorted $Bim^{-/-}$ DP thymocytes survive 10-30 fold better than wild type (WT) DP thymocytes. The pro-apoptotic Bcl-2 family members Bax and Bak are the major cell death executors in the intrinsic apoptotic pathway. Bax and Bak may participate in death by neglect as $Bax^{-/-}Bak^{-/-}$ thymocytes are resistant to spontaneous apoptosis *in vitro* [36]. After four days of *in vitro* culture, almost 100% $Bax^{-/-}Bak^{-/-}$ thymocytes survive while less than 10% WT thymocytes survive [36]. However, $Apaf1^{-/-}$ and caspase 9 $^{-/-}$ thymocytes do not have any defects in death by neglect [135]. These data suggest that the apoptosome is not essential for apoptosis in thymocytes. Compared with apoptosome, Bcl-2 family members have a broader function of controlling apoptosis and caspase activation [135]. In summary, the Bcl-2 family members $Bcl-x_L$, Bim, Bak and Bax control DP thymocyte survival and may participate in death by neglect process.

SP thymocytes and negative selection

Negative selection deletes thymocytes expressing high affinity TCRs for MHC/self peptide complexes to minimize the risk of autoimmunity in the periphery. A subpopulation of SP thymocytes may be subjected to negative selection. While the intrinsic apoptotic pathway is the major mechanism for negative selection, the extrinsic death pathway may also be involved.

After successful positive selection, DP thymocytes further differentiate into CD4 $^{+}$ or CD8 $^{+}$ single positive thymocytes and undergo several maturation steps including the downregulation of HSA and upregulation of CCR7 [136]. SP thymocytes migrate from the cortex into the medulla of the thymus. CCR7 signal is required for SP thymocyte migration into the thymic medullary region as in CCR7 or CCR7 ligand deficient mice, mature SP thymocytes accumulate in the cortex instead of medulla [137]. Recently, we have shown that anti-apoptotic protein c-FLIP is required for SP thymocyte maturation by providing protection against apoptosis [138]. Therefore, one or more death receptors may participate in the process of SP

thymocyte maturation.

Negative selection is a critical check point during thymocyte development [139, 140]. In most high-affinity TCR transgenic mice that express both TCR α and TCR β from the DN stage, massive negative selection occurs in DP cells or earlier in thymic cortex. This is seen in HY TCR transgenic mice [141]. However, when the HY TCR α chain is not expressed until the DP stage, the deletion of HY thymocytes in male mice occurs at the CD8 $^{+}$ SP stage [142]. Thus in a physiologic situation, it has been suggested that negative selection occurs during the DP to SP transition [139, 140]. For MHC class II restricted thymocytes, semi-mature thymocytes (HSA $^{+}$ CD4 $^{+}$ CD8 $^{-}$) are highly sensitive to TCR-induced apoptosis and subjected to negative selection [143]. For MHC class I restricted thymocytes, whether negative selection also occurs in the HSA $^{+}$ population remains to be determined.

Consistently, CD3 Med SP cells are subjected to negative selection. CD3 Med CD4 $^{+}$ or CD3 Med CD8 $^{+}$ SP cells contain the precursors of CD3 high SP cells. Sorted CD3 Med SP cells differentiate into CD3 high SP cells in reaggregated thymic organ culture [144]. Compared with their CD3 high SP counterparts, CD3 Med SP cells are more sensitive to apoptotic stimuli *in vitro* and contain a significant higher percentage of thymocytes expressing forbidden V β^{+} TCRs reactive with endogenous superantigen [144]. These data suggest that CD3 Med SP thymocytes are subjected to negative selection.

Furthermore, it has been suggested that negative selection mainly occurs in thymic medulla where the two major populations of antigen presenting cells (dendritic cells and medullary thymic epithelial cells) reside [145]. SP thymocytes mainly reside in thymic medulla. Therefore, SP thymocytes may be the major population subjected to negative selection.

Previous research has suggested that the extrinsic apoptotic pathway might participate in negative selection. Death receptor signaling (Fas-FasL) has been shown to play a critical role in the negative selection of semi-mature thymocytes when TCRs receive strong signals [146]. However, Fas-FasL interactions are not required for the deletion of semi-mature thymocytes when TCR stimulation is moderate.

Besides Fas-FasL interactions, other death receptors may also be involved in negative selection. However, the available results are conflicting. Anti-TNF α antibody rescues CD4 $^{+}$ SP thymocytes which otherwise undergo negative selection in fetal thymic organ culture (FTOC) [147]. TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ thymocytes undergo normal negative selection [148, 149]. However, TNFR1 $^{-/-}$ TNFR2 $^{-/-}$ double deficient thymocytes exhibit a defect in negative selection in an *in vitro* antigen presenting cell co-culture

system [150]. Therefore, the *in vivo* role of TNFR1 and TNFR2 in negative selection is unclear at present. Studies on tumor necrosis factor related apoptosis inducing ligand (TRAIL) deficient or TRAILR^{-/-} thymocytes have also generated contradictory conclusions [151-154]. In one study, TRAIL deficiency leads to a defect in anti-CD3 induced thymocyte apoptosis both *in vivo* and *in vitro* [152]. On a TRAIL^{-/-} background, DO11.10 TCR transgenic mice are resistant to OVA mediated *in vivo* thymocyte deletion [152]. When backcrossed to BALB/C, TRAIL^{-/-} mice also display a defect in the deletion of thymocytes expressing TCRs reactive with endogenous superantigen [152]. In another study, TRAIL^{-/-} thymocytes do not have any defects in anti-CD3 mediated thymocyte apoptosis *in vitro* [151]. Furthermore, TRAIL^{-/-} thymocytes display a normal deletion in response to superantigen in FTOC. When backcrossed onto a BALB/C background, TRAIL^{-/-} mice do not have any defects in the deletion of thymocytes reactive with endogenous superantigen [151]. The explanation for the discrepancy is unknown. Death receptor 3 (DR3) deficient mice have a subtle defect in thymocyte negative selection [155]. DR3^{-/-} mice display a mild defect in DP thymocyte deletion in male H-Y mice and DR3^{-/-} thymocytes show a 10% lower apoptotic rate in response to anti-CD3 stimulation *in vitro* compared with WT mice [155]. While these results suggest that the extrinsic death pathway may be involved in negative selection, further evidence is required.

However, there is strong evidence to support that the intrinsic apoptotic pathway is involved in thymocyte negative selection. The pro-apoptotic Bcl-2 family member Bim is required for normal negative selection in thymocytes as Bim^{-/-} thymocytes are resistant to negative selection in several widely used negative selection models [69]. Bim^{-/-} thymocytes are resistant to anti-CD3 induced apoptosis both *in vitro* and *in vivo* [69]. Furthermore, Bim^{-/-} thymocytes are resistant to superantigen induced deletion in FTOC [69]. Bim deficiency rescues OVA peptide induced thymocyte apoptosis in OT-II transgenic mice and thymocyte deletion in male H-Y transgenic mice [69]. Bim deficiency rescues sorted semi-mature thymocytes from anti-CD3 induced apoptosis *in vitro* [156]. These authors also presented data to rule out a possible role of Fas-FasL interaction in the TCR/CD3 induced apoptosis of semi-mature thymocytes *in vitro* [156].

The pro-apoptotic Bcl-2 family members Bax and Bak may participate in negative selection as well. Bax^{-/-}Bak^{-/-} thymocytes are highly resistant to anti-CD3 induced thymocyte apoptosis *in vitro* and endogenous superantigen induced thymocyte deletion *in vivo* [36]. However, Apaf-1^{-/-} thymocytes have no defect in negative selection [157]. Pan-caspase inhibitor p35 T cell specific transgenic mice

led to contradictory results in terms of negative selection [158, 159]. In one study, the p35 transgene rescues specific peptide and superantigen induced thymocyte apoptosis in F5 TCR transgenic mice *in vivo* [158]. p35 transgenic thymocytes are also resistant to anti-CD3 induced apoptosis *in vitro* [158]. However, in another study, the p35 transgene could only rescue 7-10% thymocytes from anti-CD3 induced apoptosis *in vitro* [159]. Furthermore, the p35 transgene has no effect on thymocyte deletion in H-Y and OT-1 transgenic mice [159]. The reason for the discrepancy is not clear at present. In general, Bcl-2 family members Bim, Bax and Bak are required for thymocyte negative selection.

In summary, several critical check points exist during thymocyte development to ensure that only thymocytes bearing intermediate affinity TCRs towards self-MHC-peptide complexes survive and further differentiate into mature T lymphocytes. Those with useless TCRs do not receive a positive selection signal and undergo death by neglect. Those with high affinity TCRs are also eliminated by negative selection. It is estimated that about 95% T lymphocyte precursors undergo apoptosis during T cell development [5]. As mentioned before, several apoptosis-related molecules play critical roles at different stages during T lymphocyte development. Therefore, apoptosis is one of the most fundamental processes underlying the development of a functional T cell repertoire.

T LYMPHOCYTE FUNCTION AND APOPTOSIS

Mounting an efficient immune response upon antigen challenge is the primary function for T lymphocytes. However, maintaining T lymphocyte homeostasis between antigenic challenges is also essential. The regulation of T cell immune responses and homeostasis has been the subject of extensive investigation. Costimulatory molecules are required for efficient T cell responses. IL-7 signal is essential for maintaining the homeostasis of both naïve and memory T cell populations. Both intrinsic and extrinsic apoptotic pathways are actively involved in the regulation of T cell responses and homeostasis. Several anti-apoptotic molecules have been identified as critical effector molecules downstream of IL-7 and costimulatory molecule signaling in mature T lymphocytes.

Maintenance of naïve T cells

Naïve T cells are long-lived resting cells, constantly circulating through blood, lymph, and secondary lymphoid organs including spleen, lymph nodes (LNs) and Peyer's Patches (PPs). In a healthy adult animal, naïve T cells do not undergo massive proliferation and T cell numbers remain constant. However, if transferred into a T cell depleted host, naïve T cells undergo homeostatic driven pro-

liferation due to the ability of T cells to respond to empty niches in the environment. In other words, T cells can sense the extra survival signals in the host. During homeostatic driven proliferation, naïve T cells gain some phenotypic markers of effector/memory T cells, including the upregulation of CD44 and downregulation of CD62L, and they even temporarily obtain some effector functions [162].

Naïve T cells require active signals, which mainly include TCR/MHC and IL-7 signals, to survive and to undergo homeostatic driven proliferation. Molecules of the intrinsic apoptotic pathway are the major effectors downstream of TCR/MHC and IL-7 signals regulating naïve T cell survival [163, 164]. Transferring naïve T cells into a MHC^{-/-} or IL-7^{-/-} host results in the rapid loss of transferred T cells [163, 165-170]. The MHC molecules delivering survival signals to naïve T cells are believed to be the same type of MHC/self-peptide molecules delivering positive selection signals in the thymus. Whether the peptides are identical to the peptides presented in the thymus during positive selection is still under debate [171-173]. However, it is generally agreed that Bcl-2 protein family may be one of the major effector molecules downstream of TCR/MHC and IL-7 signaling to control naïve T cell survival [163, 164].

T cell immune response

When a naïve T cell encounters its cognate antigen presented by a mature dendritic cell, the T cell is activated [174, 175]. Mature dendritic cells not only express high levels of MHC molecules on their surface, but also express costimulatory molecules required for T cell activation including CD80/86, OX40L, 4-1BBL and CD70. Activated T cells upregulate the expression of IL-2R and IL-2, which is the critical pro-proliferative cytokine during T cell activation. Activated T cells undergo vigorous proliferation and differentiate into effector T cells—known as T helper (T_H) cells for CD4⁺ and cytotoxic T lymphocytes (CTLs) for CD8⁺ T cells. Effector CD4⁺ and CD8⁺ T cells secrete inflammatory cytokines, and CTLs directly kill infected cells. In acute infection models, the infection is usually resolved in several days. After the infection is resolved, more than 90% of the effector T cells undergo rapid apoptosis. The remaining cells further differentiate into a memory population, which provides vigorous and efficient immune protection when antigen is re-encountered.

Effector CD4⁺ T cells perform diverse functions during an immune response. First, effector CD4⁺ T cells activate antigen presenting cells (APC) by CD40-CD40L interactions. Second, effector CD4⁺ T cells secrete cytokines to promote a B lymphocyte response. Finally, effector CD4⁺ T cells provide help for CD8⁺ T cell re-

sponses [176, 177]. It has been shown that without CD4⁺ T cell help, there is a defect in the maintenance of CD8⁺ memory T cells [178-180]. Without CD4⁺ T cells, CD8⁺ memory T cells have decreased IL-7R α expression and gradually disappear over time [181, 182]. Moreover, compared with those CD8⁺ T cells primed with CD4⁺ T cell help, the CD8⁺ T cells primed without CD4⁺ T cell help (helpless CD8) express a decreased level of Bcl-2, Bcl-x_L and c-FLIP, and an increased level of FasL and TRAIL upon secondary challenge [183]. Specifically, TRAIL mediated death receptor signaling is critical for the elimination of helpless CD8⁺ T cells during secondary expansion [183].

Both CD4⁺ and CD8⁺ T cell immune responses can be divided into three phases: expansion phase, contraction phase and memory phase. Since more *in vivo* research has been done on the CD8⁺ T cell immune response, we will mainly focus on CD8⁺ T cells during the following discussion.

EXPANSION PHASE OF CD8⁺ T CELL IMMUNE RESPONSE

After CD8⁺ naïve T cells encounter cognate antigen presented by mature DCs, CD8⁺ T cells are activated and undergo up to 3-4 rounds of cell division per day. Antigen specific CD8⁺ T cells can amplify more than 1,000 fold in a few days [174, 175].

Multiple groups have independently discovered that the CD8⁺ T cell immune response is programmed and can be triggered by a brief contact with antigen. By treating animals with antibiotics after bacterial infection to limit bacteria persistence *in vivo* or transferring CD8⁺ T cells into an antigen-free host after infection, it has clearly been demonstrated that during an acute infection, 24 hours of antigen exposure *in vivo* is enough to initiate a CD8⁺ T cell immune response. After this short exposure, CD8⁺ T cells will undergo 7-10 rounds of cell division and generate fully functional CD8⁺ effector T cells in the absence of additional antigen stimulation [184-188]. Furthermore, *in vivo* antigen presentation after acute bacterial infection is transient. During *Listeria monocytogenes* infection, *in vivo* antigen presentation lasts less than 3 d. If naïve CD8⁺ T cells are transferred more than 3 d after infection, most naïve CD8⁺ T cells do not undergo cell division *in vivo* [189].

Although a brief antigen exposure is sufficient to trigger a fully functional CD8⁺ T cell response, the magnitude at the peak of that response is directly correlated with the dose of initial infection. This correlation may reflect the ability of a larger infection to recruit more naïve antigen specific CD8⁺ T cells to the immune response [184].

During the expansion phase of a CD8⁺ T cell response,

CD8⁺ T cells undergo a series of differentiation events to remodel their gene expression profiles. CD8⁺ T cells gain the expression of effector molecules such as interferon γ (IFN γ), perforin and granzyme B. CD8⁺ T cells downregulate CD62L and CCR7 while upregulating sphingosine-1-phosphate receptor 1(S1P) to facilitate migration out of secondary lymphoid organs. Furthermore, effector CD8⁺ T cells upregulate tissue specific chemokine receptors so that they can directly migrate into the infected tissue [175, 190].

Interestingly, the effector molecule perforin negatively regulates the expansion phase of the CD8⁺ T cell response [191,192]. Infection of perforin deficient mice with *L. monocytogenes* results in a 3-4 fold increase in CD8⁺ T cell expansion compared with WT mice [192]. This increase of CD8⁺ T cell expansion in perforin deficient mice is not due to an increased bacteria load *in vivo* as immunizing perforin deficient mice with peptide loaded dendritic cells leads to identical results [192].

In summary, a brief antigen exposure is sufficient to trigger naïve CD8⁺ T cells to differentiate into CD8⁺ effector T cells. The dose of initial infection controls the magnitude of the peak of CD8⁺ T cell response.

CONTRACTION PHASE OF CD8⁺ T CELL IMMUNE RESPONSE

After the peak of the CD8⁺ T cell immune response, 90-95% of the effector cells are eliminated to maintain homeostasis in the T lymphocyte compartment. It is interesting that the kinetics of contraction phase is very similar regardless of infection model and T cell antigen specificity. The size of the surviving cell population after contraction phase correlates with the size of the CD8⁺ T cell population at the peak of expansion [193].

The role of both the extrinsic and intrinsic apoptotic pathway in CD8⁺ T cell contraction phase has been investigated. For the extrinsic apoptotic pathway, it has been found that *in vitro* activated T cells become highly sensitive to TCR-restimulation induced cell death via Fas and TNFR1 after extended culture in IL-2 [90]. However, naïve T cells are highly resistant to death receptor induced apoptosis. It is now clear that IL-2 delivers a critical sensitization signal to activated T cells and downregulates the apoptosis inhibitor c-FLIP [194, 195]. However, the *in vivo* contraction phase of the CD8⁺ T cell immune response after an acute infection is intact in the absence of death receptor signaling [196, 197]. Moreover, *in vivo* administration of high doses of the pan-caspase inhibitor zVAD has little effect on the contraction phase of the CD8⁺ immune response, while Fas induced liver injury is largely abolished [198]. These *in vivo* data argue against the role of the extrinsic death pathway in the contraction phase

during the CD8⁺ T cell immune response. Therefore, it is generally believed that activation induced cell death (AICD) is not involved in acute infection because most T cells will not re-encounter antigen several days after priming. Instead, AICD may be more relevant to chronic infection or autoimmunity *in vivo*.

The other pathway that may be involved in the contraction phase is the mitochondria-dependent intrinsic death pathway. After the peak of the CD8⁺ T cell response, when the infection has been resolved, growth or survival factors for effector CD8⁺ T cells become limited. Therefore, effector cells undergo cytokine-deprivation induced apoptosis. Bcl-2 family members are the major effectors controlling cytokine-deprivation induced apoptosis in T cells. *In vitro* studies have shown that a Bcl-2 transgene rescues activated T cells from cytokine deprivation induced cell death. Bax^{-/-}Bak^{-/-} T cells are highly resistant to multiple cell death stimuli including cytokine deprivation [36]. Furthermore, in human T cells, Bim expression can be induced by TCR signaling [199]. Bim-deficient T cells are resistant to cytokine deprivation[134] and are defective in the CD8⁺ contraction phase in herpes simplex virus infection[200, 201]. Moreover, the pro-apoptotic BH-3 only protein Noxa is induced in antigen-specific CD8⁺ T cells during the early period of the contraction phase [202]. Overall, Bcl-2 family members may participate in the contraction phase during CD8⁺ T cell responses. However, a detailed mechanism is not clear at present.

Interestingly, CD8⁺ T cells deficient in the effector molecule IFN γ have a defect in the contraction phase following infection [192, 203]. However, the mechanism underlying IFN γ mediated CD8⁺ T cell contraction is unclear. Recently, by treating animals with antibiotics before bacterial infection, it has been shown that the contraction phase is controlled by early inflammation. After antibiotic treatment, the initial infection and inflammation is diminished. As a result, the peak immune response is also diminished. However, a significant population of memory cells is still generated without a contraction phase [204]. Whether early inflammation controls CD8⁺ T cell contraction phase through IFN γ is still an open question and needs further investigation.

MEMORY PHASE OF CD8⁺ T CELL IMMUNE RESPONSE

A small population of CD8⁺ T cells survive the contraction phase to differentiate into memory cells. At a cell population level, compared with antigen specific naïve T cells, the frequency of memory T cells increases. At an individual cell level, memory T cells have a lower activation threshold than naïve T cells. Therefore when antigens are reencountered, memory T cells provide more rapid and

robust immune protection.

An interesting question in memory T cell development is whether there are markers to identify a subset within the effector T cell pool that will preferentially survive the contraction phase. Alternatively, apoptosis during the contraction phase may be a completely random event. Recently, IL-7R and CD8 α have been identified as markers to identify the early precursors of memory cells within the effector cell population [205, 206]. IL-7R⁺ or CD8 α ⁺ effector T cells have higher expression levels of anti-apoptotic Bcl-2 family members when compared with their IL-7R⁻ or CD8 α ⁻ counterparts. Furthermore, IL-7R⁺ or CD8 α ⁺ effector cells preferentially survive the contraction phase and differentiate into memory cells. In CD8 α deficient mice, CD8⁺ memory T cell formation after acute infection is compromised [206]. These data suggest that CD8 α delivers a pro-survival signal to CD8⁺ memory T cell precursors. CD8 α binds to the non-classical MHC class I molecule thymic leukemia antigen (TL) [207]. Surprisingly, in a mouse strain lacking non-classical MHC class I expression (β 2m deficient mice crossed with single classical MHC I transgenic mice), CD8⁺ memory T cells develop normally. Therefore, CD8 α /TL interaction may not be required for CD8⁺ memory T cell survival and CD8 α may only serve as a marker for CD8⁺ memory T cell precursors [208]. However, whether CD8 α has an alternative ligand and whether CD8 α delivers a signal without ligand binding may need further investigation.

Memory T cells need active signals to survive. Memory T cells can persist without specific antigen and have a higher turn-over rate compared to naïve T cells in a normal host. In a lymphopenic host, memory T cells undergo homeostatic driven proliferation. It is believed that CD8⁺ memory T cells can persist without MHC class I [209]. However, CD8⁺ memory T cell survival depends on IL-15 in a normal host, and also depends on IL-7 in a lymphopenic host or in an IL-15 deficient host [169, 210-213]. CD4⁺ memory T cell survival and homeostatic proliferation depend on IL-7 signal and also require TCR/MHC class II interaction. The IL-7R signal and TCR signal have partial overlapping function in CD4⁺ memory T lymphocytes [214].

IL-7 mediated T lymphocyte survival

As discussed above, the survival of both naïve and memory CD4⁺ and CD8⁺ T cells is dependent on IL-7 signaling. The IL-7R has two subunits: an IL-7R α chain shared with thymic stromal lymphopoietin (TSLP) and a common γ_c , which is shared with IL-2, IL-4, IL-9, IL-15 and IL-21 [164].

The expression of the IL-7R is tightly regulated during T cell development and T cell immune responses. IL-7R

is expressed on the early precursors of T lymphocytes (DN stage), downregulated in ISP cells and re-expressed on mature T cells. Although after T cell activation the IL-7R is transiently downregulated, both naïve and memory T cells express IL-7R [205]. Downregulation of the IL-7R at the DP stage is required to limit the competition between DN and DP thymocytes for available IL-7 [215]. Recently, it has been suggested that besides its role in survival and proliferation, IL-7 may have functions in thymocyte differentiation. IL-7 signaling downregulates three critical transcriptional factors involved in the ISP to DP transition: LEF, TCF-1 and ROR γ [216]. These data suggest that downregulation of the IL-7R may be required for the upregulation of LEF, TCF-1 and ROR γ , and for the developmental transition from ISP to DP. In mature T cells, IL-7 and other pro-survival cytokine signaling downregulate the transcription of IL-7R α . Interestingly, in CD8⁺ T cells, the downregulation of IL-7R α by IL-7 signaling depends on the transcriptional factor Gfl-1, while in CD4⁺ T cells, it does not. It has been proposed that those T cells that have received survival signals lose the ability to compete for IL-7. Therefore, by this mechanism, the T cell pool is maximized by limited amount of IL-7 [217].

The major function of IL-7 in T lymphocytes is to enhance survival. Mediators in the intrinsic apoptotic pathway are the major effectors downstream of IL-7 signaling. It is well-known that IL-7 activates a Jak/STAT signaling pathway [218]. STAT5 activates Bcl-2 expression and a Bcl-2 transgene can rescue the defect in IL-7R^{-/-} mice [9, 10]. Similarly, a Bcl-2 transgene or a deficiency in Bax partially rescues the severe defects in lymphopoiesis in mice lacking Jak3, which is downstream of IL-7 signaling [219]. Furthermore, IL-7 signal activates PI-3K/Akt [218]. It is well established that the PI-3K/Akt pathway phosphorylates the pro-apoptotic protein Bad, which inhibits its function [68, 220]. Activated Akt represses the activity of the transcription factor FOXO, which classically activates the expression of the pro-apoptotic protein Bim [221]. Recently, another anti-apoptotic Bcl-2 family member, Mcl-1, has been shown to be essential for the survival of T and B lymphocytes. It is suggested that Mcl-1 may be an important downstream target of IL-7 signaling [24]. Therefore, IL-7 signaling activates Bcl-2 and Mcl-1 and inhibits Bad and Bim to promote T cell survival.

Costimulatory molecules and anti-apoptotic molecules in T cells

Specific TCR/MHC-peptide interactions deliver an activation signal to T cells. However, optimal T cell activation is only achieved when costimulatory signals are present. Costimulatory signals promote T cell activation and survival. In most situations, different costimulatory

molecules play functionally, spatially and temporally distinct roles in the T cell immune response. For example, compared with CD28 signals, OX40 and 4-1BB signals function in a later phase of the T cell immune response. However, different costimulatory molecules may also have overlapping functions. For instance, several costimulatory molecules, including CD28, OX40 and 4-1BB, induce the expression of pro-survival Bcl-2 family members to promote T cell survival after activation.

It is well established that CD28-CD80/CD86 signaling is crucial for T cell activation and proliferation. Because of its constitutive expression on mature T cells, CD28 is one of the most critical costimulatory molecules during the initial phase of T cell activation. *In vitro* studies have shown that CD28 signaling specifically upregulates the expression of the anti-apoptotic protein Bcl-x_L, but not Bcl-2 [222]. Point mutation analysis has shown that tyrosine residue Y170 on the cytoplasmic tail of CD28 is essential for the recruitment of PI-3K, which induces the expression of Bcl-x_L [223-225]. CD28 carrying a mutation at Y170 can promote T cell activation, proliferation and IL-2 production. However, the mutant CD28 cannot upregulate Bcl-x_L expression and cannot provide anti-apoptotic signals to T cells. CD28 deficiency has a great impact on both CD4⁺ and CD8⁺ T effector cell development and function. In CD28^{-/-} mice, basal IgG levels are greatly reduced. After infection or immunization, germinal center formation and immunoglobulin class switch are diminished [226, 227]. A very similar phenotype has been observed when two of CD28's ligands are disrupted in CD80^{-/-}CD86^{-/-} double deficient mice [228]. Effector and memory CD8⁺ T cell populations in CD28^{-/-} mice are reduced by 2-3 fold in lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* infection models [229, 230]. Furthermore, the numbers of effector CD8⁺ T cells in secondary lymphoid organs during primary and memory responses to influenza virus infection are greatly reduced in CD28^{-/-} mice [231]. Therefore, CD28 is required for both CD4⁺ and CD8⁺ T cell responses.

OX40 is an essential costimulatory molecule for CD4⁺ T cell immune responses. Expression of OX40 can be detected 48 hours after T cells receive activation signals. OX40 functions after CD28 to sustain the expression of anti-apoptotic molecules in activated CD4⁺ T cells. OX40 signaling activates the PI-3K/Akt pathway and upregulates the anti-apoptotic proteins Bcl-x_L and Bcl-2 [232]. OX40^{-/-} T cells have a defect in long term survival. This survival defect is rescued by retrovirus mediated overexpression of Bcl-2, Bcl-x_L or activated Akt kinase [232, 233]. OX40L is detected on activated B cells, macrophages and dendritic cells [234]. Interestingly, OX40L is also detected on a newly identified population of accessory cells (CD3⁺

CD4⁺MHC II⁺ OX40L⁺ B220⁻) in the spleen *in vivo* [235]. After immunization, OX40L⁺ accessory cells are in close contact with T cells [235]. Furthermore, this contact correlates with the survival advantage of activated OX40^{+/+} T cells over OX40^{-/-} T cells *in vivo* [235]. The analysis of OX40^{-/-} mice has revealed that the CD8⁺ T cell immune response during acute viral infection is not impaired. However, CD4⁺ memory T cell recall response is profoundly reduced in OX40^{-/-} mice after immunization, although germinal center formation is intact [236]. OX40^{-/-} CD4⁺ T cells can produce IL-2 and expand during the initial phase of an immune response. However, OX40^{-/-} CD4⁺ T cells have a survival defect in the later phase. These data suggest that OX40 is preferentially involved in CD4⁺ memory cell development and maintenance. Based on these observations, it has been proposed that OX40 signals function after CD28 signals to maintain the expression of anti-apoptotic proteins Bcl-x_L and Bcl-2 to promote CD4⁺ T cell survival during immune responses.

In parallel to the function of OX40 in CD4⁺ T cells, the costimulatory molecule 4-1BB promotes CD8⁺ T cell immune response presumably by induction of anti-apoptotic molecules in activated CD8⁺ T cells. 4-1BB is not expressed on resting T lymphocytes. Expression of 4-1BB is induced 24-48 h after T cell activation. 4-1BB is expressed on activated antigen presenting cells [234]. 4-1BB signal also induces the anti-apoptotic proteins Bcl-x_L and A1/Bfl-1 in T lymphocytes through an NF-κB dependent pathway [238-240]. However, during viral infection, CD4⁺ T cell function is unimpaired in 4-1BB^{-/-} mice. In contrast, effector CD8⁺ T cell response, especially CD8⁺ T cell memory response is defective in 4-1BB deficient mice [241-243]. These data suggest that 4-1BB is required for CD8⁺ effector and memory cell development and survival during viral infection. 4-1BB may function after CD28 to promote the expression of anti-apoptotic molecules in activated CD8⁺ T cells.

CD27 is another critical costimulatory molecule required for memory T cell development. CD27 has a low to moderate expression level on naïve CD4⁺ and CD8⁺ T lymphocytes [234]. After T cell activation, CD27 is quickly upregulated and downregulated again during the expansion phase of the T cell response [234]. During viral infection in both mice and humans, a CD27⁺CD8⁺ cell population has been identified as the most efficient effector cell population [244, 245]. CD27 signal not only promotes T cell division, but also T cell survival after activation *in vitro* [231]. Whether CD27 signals promote T cell survival by induction of anti-apoptotic Bcl-2 family members needs further investigation. In CD27^{-/-} mice, after intranasal influenza infection, there is a dramatic reduction of effector CD4⁺ and CD8⁺ cells in the lung and draining lymph

nodes (DLN) and a mild reduction of effector T cells in the spleen. This is true both in primary and memory responses. In contrast, in the same infection model the major defect for CD28^{-/-} mice lies in the accumulation of effector T cells in the spleen [231, 246].

In summary, costimulatory molecules are essential for T cell function. Anti-apoptotic proteins are important mediators downstream of costimulatory signals. Interestingly, although Bcl-x_L is one of the most important effector molecules downstream of CD28, OX40 and 4-1BB signals, Bcl-x_L is dispensable for the development of effector and memory T lymphocytes [247]. This result may be explained by the redundancy between different anti-apoptotic Bcl-2 family members or different anti-apoptotic pathways.

CONCLUSION

The function of apoptosis in the development and function of T lymphocytes has been an exciting field for years. However, many issues remain to be addressed. For examples, what are the detailed molecular mechanisms of thymocyte apoptosis *in vivo*? Which molecules are essential for effector and memory T lymphocyte survival? Which molecules are involved in the contraction phase of T cell immune responses? The answers to these questions will not only further our knowledge of T cell biology, but will have great implications in vaccine design and immunotherapy as well.

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