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Programmed Cell Death in T Cell Development

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1. Introduction

The mammalian immune system is a complex network of many cell types and proteins that collectively coordinate a protective response against foreign entities. The immune system can be divided into two broad categories: innate and adaptive immunity. Adaptive immunity, which is primarily mediated by T and B lymphocytes, first arose in jawed vertebrates and has several distinct features from the more ancient innate immune system (Pancer and Cooper 2006). While innate immunity is characterized by non-specific recognition of conserved molecular patterns leading to a rapid effector response, the adaptive immune response is delayed by the required expansion of lymphocytes bearing receptors specific for a particular antigen. After the primary immune response, a small fraction of activated lymphocytes remain as memory cells, which respond to subsequent encounters with the same antigen in a more rapid and robust manner.

Due to the enormous diversity of antigens to which the adaptive immune system must respond, generation of antigen receptors cannot occur on a one gene-one protein basis. Instead, diversity is achieved through recombination of genetic segments, nucleotide additions and deletions, and pairing of different chains to form the complete antigen receptor. For example, of a theoretical 10^{15} T cell receptor (TCR) specificities in humans, only a small fraction is represented by circulating T cells (Arstila et al. 1999). Most lymphocytes do not complete development and are eliminated by programmed cell death. This can occur at several checkpoints both independent and dependent of the antigen receptor specificity. T and B cell development are analogous; both ultimately require the generation of an antigen receptor that can bind self-antigen with an affinity just high enough to enable survival and maturation. Lymphocytes bearing receptors with excessively high affinity for self-antigen are eliminated. This chapter focuses on the role of programmed cell death during T cell development in the thymus. Recent advances in the field reveal a complex and elegant system designed to select for immunocompetent and self-tolerant T cells.

2. TCR-independent T cell development

T cell development occurs in the thymus, a bilobed organ composed of an outer capsule, a peripheral cortex, and a central medulla. T cell progenitors from the bone marrow or fetal liver seed the thymic cortex as double negative (DN) thymocytes, so called because they lack expression of CD4 and CD8 co-receptors. Murine DN thymocytes progress through four stages of development defined by differential expression of the proteins CD44 and CD25 (Godfrey et al. 1993) (**Figure 1**). The earliest progenitors that seed the thymus are termed DN1 (CD44⁺CD25⁻). T cell lineage commitment initiates rearrangement of the TCR β chain by recombination activating gene-1 and -2 (Rag-1 and Rag-2) enzymes at the DN2 stage (CD44⁺CD25⁺), which continues into the DN3 stage (CD44⁻CD25⁺) (Livak et al. 1999). Rearrangement of the TCR γ and δ loci are also evident in DN3 thymocytes and it is at this stage that the $\gamma\delta$ T cell lineage diverges from conventional $\alpha\beta$ T cells (MacDonald et al. 2001). In this chapter, we focus on the role of apoptosis in $\alpha\beta$ T cell development.

Figure 1. Conventional T cell development in the thymus.

T cell progenitors enter the thymus on the cortical side of the cortico-medullary junction as DN thymocytes, lacking expression of CD4 and CD8 co-receptors. DN thymocytes progress through four stages of development defined by differential expression of CD44 and CD25. Only thymocytes expressing a functional TCR β chain survive the β -selection checkpoint at the DN3 to DN4 transition and are permitted to continue development into CD4⁺CD8⁺ DP thymocytes. DP thymocytes have three fates depending on the affinity of its TCR for self-

pMHC: death by neglect, negative selection, or positive selection. Positively selected thymocytes differentiate into CD4⁺CD8⁻ or CD4⁺CD8⁺ SP cells and migrate to the medulla, where negative selection against tissue-restricted antigens occurs. SP thymocytes that survive this process enter the peripheral T cell repertoire.

While the mature $\alpha\beta$ TCR is composed of one α and β chain each, the TCR β chain is initially paired with an invariant pre-TCR α chain, together forming the pre-TCR (Saint-Ruf et al. 1994). Signaling through the pre-TCR or mature TCR is mediated by an associated protein complex that contains a CD3 γ /CD3 ϵ heterodimer, CD3 δ /CD3 ϵ heterodimer, and CD3 ζ homodimer (**Figure 2**). The TCR only recognizes peptide antigen when presented on major histocompatibility complex (MHC) molecules. A common method of artificially stimulating thymocytes in vitro is using agonist antibodies against CD3 ϵ and the co-stimulatory molecule CD28. In contrast to the mature TCR, interaction between the pre-TCR and peptide-MHC (pMHC) is not required for signaling (Irving et al. 1998). Rather, translocation of pre-TCR complexes to lipid rafts in the plasma membrane has been proposed to provide a platform for association with signaling proteins (Saint-Ruf et al. 2000). Pre-TCR signaling is required for allelic exclusion at the TCR β locus, survival, differentiation into DN4 (CD44⁺CD25⁻) thymocytes, proliferation and subsequent differentiation into CD4⁺CD8⁺ (DP) thymocytes, and initiation of TCR α rearrangement (Michie and Zuniga-Pflucker 2002). This has been demonstrated by genetic ablation of Rag-2, pre-TCR α , and numerous downstream signaling components (review of pre-TCR and TCR signaling pathways in thymocytes in references (Michie and Zuniga-Pflucker 2002; Starr et al. 2003)). Since this process selects for thymocytes with functional TCR β rearrangements, it is referred to as the β -selection checkpoint.

Rearrangement of the TCR α locus occurs at low levels in DN4 thymocytes but does not occur at full scale until the DP stage (Hernandez-Munain et al. 1999). DP thymocytes have a lifespan of 3-4 days (Egerton et al. 1990), during which time multiple recombination events can occur at each TCR α allele until the generation of an $\alpha\beta$ TCR that engages self-pMHC expressed on cortical thymic epithelial cells. The majority of DP thymocytes do not express a functional $\alpha\beta$ TCR or do not engage self-pMHC within this time window. Thymocytes that fail this self-MHC restriction checkpoint undergo “death by neglect” to maximize the utility of the T cell repertoire.

2.1. Apoptosis in pre- β -selection thymocytes

The thymic microenvironment provides signals to developing thymocytes through cell-cell interactions and cytokines. Two key cell surface receptors important for survival of pre- β -selection thymocytes are the IL-7 receptor and Notch.

IL-7 is a cytokine produced by thymic epithelial cells that signals through a receptor consisting of an IL-7R α chain and a γ_c chain, resulting in the activation of a variety of signaling pathways. IL-7 is known to promote survival and proliferation but this chapter will focus only on the role of IL-7 signaling in thymocyte survival. Early studies showed that

IL-7 increases the viability of DN1, DN2, and DN3 thymocytes cultured *in vitro* (Godfrey et al. 1993; Kim et al. 1998). *In vivo* studies using IL-7R $\alpha^{-/-}$ mice revealed a deficit of CD25⁺ DN (i.e. DN2 and DN3) thymocytes, suggesting that IL-7 is needed for the transition to and/or at these stages (Peschon et al. 1994; Maraskovsky et al. 1997). Later studies that examined sorted DN populations confirm that the DN2 and DN3 subsets have the highest expression of IL-7R α (Yu et al. 2004) and responsiveness to IL-7 stimulation (Van De Wiele et al. 2004). IL-7 promotes thymocyte survival through both positive regulation of anti-apoptotic members and negative regulation of pro-apoptotic members of the B cell lymphoma (Bcl) 2 family (for a summary of the role of Bcl-2 family members in T cell development, refer to **Table 1**). For example, IL-7 stimulation of CD3 ϵ^{-} DN thymocytes induces expression of the anti-apoptotic protein Bcl-2 (von Freeden-Jeffry et al. 1997; Kim et al. 1998) and overexpression of Bcl-2 in IL-7R $\alpha^{-/-}$ (Maraskovsky et al. 1997; Khaled et al. 2002) or $\gamma\epsilon^{-/-}$ (Kondo et al. 1997) mice is sufficient to rescue development. Additionally, myeloid cell leukemia sequence 1 (Mcl-1), another anti-apoptotic Bcl-2 family member, is induced by IL-7 and can promote DN2 survival (Opferman et al. 2003). While a role for the pro-apoptotic protein Bcl-2 homologous antagonist/killer (Bak) is controversial (Khaled et al. 2002; Dunkle et al. 2010), a role for Bcl-2-associated X protein (Bax) in death by IL-7 starvation is more established. IL-7 stimulation of DN thymocytes reduces Bax expression, while IL-7 withdrawal causes mitochondrial translocation of Bax in a DN2-derived cell line called D1 (Kim et al. 1998; Khaled et al. 1999). Furthermore, Bax deficiency partially rescues DN thymocyte death in IL-7R $\alpha^{-/-}$ mice (Khaled et al. 2002). IL-7 signaling is thought to promote DN thymocyte survival in part due to activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. Akt is activated following recruitment to the plasma membrane molecule phosphatidylinositol (3,4,5)-triphosphate (PIP₃), whose production is mediated by PI3K and inhibited by phosphatase and tensin homolog (PTEN) (Song et al. 2005). Consistent with high IL-7 responsiveness, DN2 thymocytes are especially sensitive to death when treated with PI3K inhibitor (Khaled et al. 2002). One substrate of Akt is the pro-apoptotic protein Bcl-2-associated death promoter (Bad), which is sequestered in the cytosol when phosphorylated by Akt. IL-7 stimulation of DN2 and DN3 thymocytes increases Bad phosphorylation, which in D1 cells was shown to be Akt-dependent (Khaled et al. 2002; Li et al. 2004). In contrast, IL-7 withdrawal results in translocation of Bad to mitochondria, where it inhibits Bcl-2 to promote Bax activation. While the pro-apoptotic Bcl-2 member Bcl-2-interacting mediator of cell death (Bim) is another known target of Akt, Bim expression in DN thymocytes is not regulated by IL-7 and Bim deficiency cannot rescue the DN2 and DN3 blocks in adult IL-7R $\alpha^{-/-}$ mice (Khaled et al. 2002; Pellegrini et al. 2004). Additionally, IL-7 has been shown to promote survival of immature (CD34⁺) human thymocytes through activation of the PI3K/Akt pathway, though this marker does not distinguish between pre- and post- β -selection DN cells (Pallard et al. 1999). Taken together, these results indicate that IL-7 is critical for pre- β -selection thymocyte survival largely through regulation of Bcl-2 family members and the intrinsic apoptosis pathway. Consistent with this, DN death in the absence of IL-7 is characterized by DNA fragmentation and annexin V binding (Kim et al. 1998). However, the caspase inhibitors z-VAD and z-DEVD abrogate DNA fragmentation but do not prevent death, indicating the contribution of a non-apoptotic cell death pathway.

In addition to producing cytokines, thymic epithelial cells regulate thymocyte development through direct interactions. Interactions between Notch proteins and ligands of the Delta-like and Jagged families are critical at several stages of T cell development. In mice, ligand binding induces proteolytic release of the Notch intracellular domain, which translocates to the nucleus and activates the transcriptional regulator recombination signal binding protein for immunoglobulin kappa J region (RBP-J), leading to transcription of target genes. Inducible deletion of Notch1 or RBP-J in bone marrow precursors results in loss of thymocytes as early as the DN1 stage and development of thymic precursors into B cells (Wilson et al. 2001; Han et al. 2002). While Notch1 is critical for early T cell lineage commitment, little is known about the role of Notch1 in survival of pre- β -selection thymocytes. Delta-like4 is the non-redundant Notch1 ligand required for T cell development *in vivo* (Hozumi et al. 2008; Koch et al. 2008) but ectopic expression of either Delta-like1 or Delta-like4 on OP9 bone marrow-derived stromal cells can support T cell development *in vitro* (Schmitt and Zuniga-Pflucker 2002; Hozumi et al. 2004). Culture of pre- β -selection murine (Rag2^{-/-}) or human (CD3 ϵ ⁻) DN thymocytes in the presence of Delta-like1 or Delta-like4 results in a decreased frequency of cell death (Ciofani et al. 2004; Magri et al. 2009). Notch1 promotes survival of Rag2^{-/-} DN3 thymocytes by maintaining glucose metabolism in an Akt-dependent manner (Ciofani and Zuniga-Pflucker 2005). Thus, it is hypothesized that Notch1 signaling generates a metabolic environment permissive for β -selection. More work is needed to characterize the anti-apoptotic aspect of Notch1 signaling during early T cell development *in vivo*.

2.2. Life and death at the β -selection checkpoint

While IL-7 is critical for T cell development up to the DN3 stage, several studies have found little to no role for IL-7 signaling in thymocyte survival during β -selection. This is due to the fact that post- β -selection DN3 and DN4 thymocytes have decreased expression of IL-7R α and subsequently reduced responsiveness to IL-7 compared to pre- β -selection stages (Van De Wiele et al. 2004; Yu et al. 2004; Van de Wiele et al. 2007; Teague et al. 2010). Interestingly, transgenic IL-7R α expression ultimately impairs differentiation into DP thymocytes, suggesting that progression through β -selection is impeded by IL-7 signaling (Yu et al. 2004).

Thymocyte survival during β -selection critically depends on signals downstream of the pre-TCR. Consistent with this, activation of caspase-3 is apparent in Rag1^{-/-} DN3 thymocytes and is abrogated upon anti-CD3 ϵ stimulation (Mandal et al. 2005). In contrast to its role in promoting survival of pre- β -selection DN3 thymocytes, Notch1 signaling has been proposed to eliminate cells that fail to rearrange the TCR β chain (Wolfer et al. 2002). Interestingly, Notch1 expression is induced by the transcription factor E2A, which is also implicated in elimination of TCR β ⁻ thymocytes (Michie and Zuniga-Pflucker 2002). In support of this, E2A^{-/-} mice develop lymphoma and ectopic expression of E2A induces death but not cell cycle arrest in lymphoma cells (Engel and Murre 1999). Pre-TCR signaling negatively regulates E2A activity and Notch1 expression, which may be one mechanism of promoting survival of thymocytes that express functional TCR β chains (Yashiro-Ohtani et al. 2009).

Contrary to these reports, constitutive Notch1 activity in Rag2^{-/-} mice does not increase thymocyte apoptosis before or after anti-CD3 ϵ stimulation, arguing against the idea that Notch1 promotes death of TCR β ⁻ cells during β -selection (Huang et al. 2003). Thus, the role of Notch1 in thymocyte survival at the β -selection checkpoint requires further clarification.

As in pre- β -selection thymocytes, the PI3K/Akt pathway plays an important role in thymocyte survival during β -selection. Pre-TCR signaling during β -selection induces Akt activation in a Notch1-independent manner (Ciofani and Zuniga-Pflucker 2005; Mao et al. 2007). Mice deficient in PI3K or Akt show a partial block at the DN3 to DN4 transition, while deletion of PTEN or expression of constitutively active Akt rescues β -selection in Rag2^{-/-} mice (Rodriguez-Borlado et al. 2003; Hagenbeek et al. 2004; Mao et al. 2007). In PTEN-deficient Rag2^{-/-} mice, unchecked Akt activation allows the survival and expansion of abnormal TCR β ⁻ cells (Hagenbeek et al. 2004). Conversely, deletion of Akt results in increased DN thymocyte death (Juntilla et al. 2007; Mao et al. 2007). Activated Akt controls the expression of several downstream apoptotic proteins. For example, through phosphorylation and nuclear exclusion of the transcription factor forkhead box O3a (FoxO3a), pre-TCR-induced Akt promotes transcriptional downregulation of Bim (Mandal et al. 2008). Survival during β -selection critically depends on pre-TCR-induced inactivation of the tumour suppressor p53, which would normally induce apoptosis in response to double-stranded DNA breaks such as those caused by TCR β recombination (Jiang et al. 1996; Haks et al. 1999). Using pre-TCR α ^{-/-} DN3 thymocytes, which can undergo TCR β recombination but not pre-TCR signaling, it was found that p53 expression remains high and directly activates transcription of the pro-apoptotic protein Bcl-2 homology 3 interacting-domain death agonist (Bid) (Mandal et al. 2008). As demonstrated in other cell types, Akt may promote murine double minute 2 (Mdm2)-mediated ubiquitination of p53 during β -selection (Ogawara et al. 2002). In support of Bim and Bid as executioners of apoptosis during β -selection, deletion of either Bim or Bid in pre-TCR α ^{-/-} mice significantly reduces the percentage of apoptotic DN3 thymocytes. However, in contrast to constitutive Akt activity, Bim or Bid deficiency does not allow further development to the DP stage (Hagenbeek et al. 2004; Mao et al. 2007; Mandal et al. 2008). Thus, downregulation of Bim and Bid are important for survival at the β -selection checkpoint, but additional signals from the pre-TCR are needed for differentiation and proliferation.

In addition to pre-TCR-mediated regulation of Bim and Bid through Akt activation, pre-TCR signaling also upregulates the anti-apoptotic protein Bcl2A1 but not Bcl-2, Mcl-1, or Bcl-xL (Mandal et al. 2005; Trampont et al. 2010). Pre-TCR induction of Bcl2A1 appears to be mediated by the protein kinase C (PKC) pathway and is independent of Akt (Mandal et al. 2005). Retroviral expression of Bcl2A1 in Rag1^{-/-} thymocytes promotes their survival and differentiation *in vivo*, whereas knockdown of Bcl2A1 increases apoptosis of pre-TCR⁺ but not pre-TCR⁻ cell lines (Mandal et al. 2005). More recently, signaling through the chemokine receptor C-X-C motif receptor 4 (CXCR4) has been reported to provide co-stimulatory signals to the pre-TCR, converging on activation of extracellular signal-regulated kinase (ERK) 1/2 (Trampont et al. 2010). A role for ERK1/2 signaling in β -selection is established but its contribution to survival is not well understood (Michie and Zuniga-Pflucker 2002).

Deletion of CXCR4 impaired thymocyte survival during β -selection and this was at least due to decreased Bcl2A1 expression, implicating the ERK1/2 pathway as another regulator of its expression (Tramont et al. 2010).

The extrinsic apoptosis pathway initiated by death receptor signaling may also play a role at the β -selection checkpoint. Transgenic expression of a dominant-negative form of Fas-associated death domain (FADD), an adaptor molecule required for apoptosis induction downstream of multiple death receptors, partially rescued thymocyte development in Rag1^{-/-} mice (Newton et al. 2000). Though Fas has been ruled out, it remains unclear which death receptors are involved.

Collectively, both inactivation of pro-apoptotic factors and induction of anti-apoptotic factors contribute to thymocyte survival during β -selection. Regulation of the Bcl-2 family by the PI3K/Akt, PKC, and ERK1/2 pathways are important mechanisms utilized by the pre-TCR to mediate survival of thymocytes at the β -selection checkpoint.

2.3. Death by neglect

Successful β -selection initiates rearrangement of the TCR α locus and differentiation into DP thymocytes (**Figure 1**). The number of recombination events correlates with the lifespan of the cell. Long-lived DP thymocytes exhaust the TCR α locus, thereby maximizing the chance of producing a functional TCR and engaging a positively selecting pMHC (Guo et al. 2002). Yet this is a relatively rare fate; most DP thymocytes undergo death by neglect upon failure to receive survival signals. This section discusses the factors that control DP survival in the absence of signaling through Notch1, IL-7 receptor, and the TCR (Huang et al. 2003; Yu et al. 2004). A prominent theory in the past was that glucocorticoids produced by thymic epithelial cells induce death by neglect in DP thymocytes that have not received TCR-induced resistance (Cohen 1992; Vacchio et al. 1994). However, studies in mice with hematopoietic-specific glucocorticoid receptor deficiency suggest that glucocorticoids do not have a significant role in death by neglect (Brewer et al. 2002; Purton et al. 2002).

Though it has its limitations, measuring spontaneous thymocyte death *in vitro* is a common way to study death by neglect. The extrinsic apoptosis pathway does not appear to play a significant role as blocking Fas/Fas ligand interactions or expression of dominant-negative FADD in thymocytes does not impair spontaneous death (Newton et al. 1998; Zhang et al. 2000). In contrast, many factors involved in the intrinsic apoptosis pathway are implicated in death by neglect. For example, DP thymocytes deficient for the pro-apoptotic proteins Bim or p53-upregulated modulator of apoptosis (Puma) have increased viability *in vitro* compared to wildtype DP, and deletion of both Bim and Puma further improves survival (Bouillet et al. 1999; Erlacher et al. 2006). In addition, Bax^{-/-} Bak^{-/-} thymocytes are resistant to spontaneous death (Rathmell et al. 2002). Of the anti-apoptotic Bcl-2 family members, numerous studies have shown that Bcl-xL plays a critical role in counteracting death by neglect. For one, the expression pattern of Bcl-xL strongly suggests that it promotes survival during the TCR-independent DP phase since spontaneous death over time correlates with

decreased Bcl-x_L levels (Zhang et al. 2000), and Bcl-x_L expression is mostly restricted to DP thymocytes *in vivo* (Ma et al. 1995). Furthermore, deletion of the Bcl-x gene renders DP thymocytes more susceptible to apoptosis *in vitro* (Ma et al. 1995; Zhang and He 2005). Deletion of Mcl-1 also impairs DP thymocyte survival *in vitro* (Dzhagalov et al. 2008). While Mcl-1 deficiency results in a more modest loss of DP thymocytes *in vivo* compared to Bcl-x_L deficiency, combined deletion of Mcl-1 and Bcl-x_L results in a severe reduction in DP numbers, suggesting that both factors are important in preventing death by neglect.

The transcription factor retinoic acid-related orphan receptor (ROR) γ t is a key activator of Bcl-x_L expression in DP cells. Similar to Bcl-x_L deficiency, deletion of ROR γ t results in increased DP apoptosis *in vivo* and reduced DP viability *in vitro* (Sun et al. 2000). Impaired DP survival in the absence of ROR γ t is likely due to reduced Bcl-x_L expression as complementation of ROR γ t^{-/-} mice with transgenic Bcl-x_L rescues survival (Sun et al. 2000). Furthermore, decreased processivity of the TCR α locus is found in ROR γ t^{-/-} mice, while the opposite is true for Bcl-x_L transgenic mice, highlighting the importance of these survival factors to the generation of a functional and self-restricted TCR (Guo et al. 2002). Pre-TCR signaling upregulates ROR γ t expression; however, ROR γ t activity is temporarily inhibited to allow generation of a large DP pool following β -selection. Active ROR γ t inhibits the cell cycle and promotes survival of resting DP thymocytes (Xi and Kersh 2004; Xi et al. 2006). Consistent with the importance of ROR γ t and Bcl-x_L in preventing death by neglect, multiple pathways have been reported to regulate their expression. For example, pre-TCR signaling induces expression of the transcription factor T cell factor 1 (TCF-1), which associates with β -catenin to execute Wnt signaling (Goux et al. 2005). TCF-1^{-/-} thymocytes have reduced expression of ROR γ t and Bcl-x_L (Yuan et al. 2010); only TCF-1 isoforms that can interact with β -catenin are able to induce Bcl-x_L expression and rescue survival of TCF-1^{-/-} DP thymocytes (Ioannidis et al. 2001). These data suggest that pre-TCR signaling and Wnt signaling cooperate to promote DP survival. Contrary to these reports, stabilization of β -catenin has been shown to impair DP survival (Gounari et al. 2001). Since β -catenin provides the transactivation domain and is thus the limiting factor in β -catenin/TCF-1-mediated transcription, a possible explanation is that physiological TCF-1 and β -catenin interactions promote survival while constitutively active signaling triggers tumour suppressors to induce apoptosis. The PI3K/Akt pathway reprises its role as a key mediator of thymocyte survival by also opposing death by neglect. Expression of constitutively active Akt enhances DP thymocyte survival in media and in fetal thymic organ cultures, while ablation of Akt or PI3K results in increased DP apoptosis (Jones et al. 2000; Swat et al. 2006; Mao et al. 2007). Specifically, the isoforms Akt1, Akt2, and PI3K δ are implicated in DP survival, with the role of PI3K γ ^{-/-} being more controversial (Sasaki et al. 2000; Swat et al. 2006; Mao et al. 2007). Bcl-x_L induction is at least part of the mechanism by which Akt promotes DP survival (Jones et al. 2000). Interestingly, Akt is a negative regulator of glycogen synthase kinase 3 (GSK3), a kinase that inhibits Wnt signaling through destabilization of β -catenin (Gounari et al. 2001; Song et al. 2005). Thus, in addition to upregulation of TCF-1, pre-TCR signaling may synergize with the Wnt pathway through Akt-mediated stabilization of β -catenin.

Promotion of glucose uptake and glycolysis by Akt in pre- β -selection DN3 thymocytes was previously mentioned (Ciofani and Zuniga-Pflucker 2005). Unlike DN thymocytes, only a small fraction of DP express glucose transporter 1 and it is unknown whether Akt regulates its expression (Swainson et al. 2005). However, conservation of energy and enhancement of energy production do appear to play an important role in extending the lifespan of DP thymocytes. It was recently reported that liver kinase B1, which activates ADP-activated protein kinase (AMPK) in response to ATP depletion, promotes DP survival *in vivo* and *in vitro* (Cao et al. 2010). Activated AMPK enacts metabolic changes to promote cell survival, and in DP thymocytes, its mechanism appears to include ROR γ t and Bcl-xL expression (Cao et al. 2010). Aside from TCF-1 and ROR γ t, the transcription factor c-myc is also thought to induce Bcl-xL expression in DP thymocytes in a TCF-1 and ROR γ t-independent manner (Yuan et al. 2010).

Taken together, these studies show that Mcl-1 and Bcl-xL play critical roles in preventing death by neglect of DP thymocytes, in part due to antagonism of pro-apoptotic Bcl-2 family members. Despite involvement of the intrinsic apoptosis pathway in death by neglect, components of the apoptosome, caspase-9 and apoptotic protease-activating factor 1 (Apaf-1), have been found to be mostly dispensable in spontaneous thymocyte death (Marsden et al. 2002). It was proposed that spontaneous death in the absence of caspase-9 and Apaf-1 is mediated by a low level of active caspase-7. However, another study found that z-DEVD, an inhibitor with preference for caspases-3 and -7, does not inhibit spontaneous thymocyte apoptosis (Zhang et al. 2000). Both studies reported that pan-caspase inhibitors such as z-VAD and IDN-1965 partially block spontaneous death. While pharmacological inhibitors have limitations, these data suggest the possible involvement of other caspases and/or caspase-independent cell death mechanisms.

3. TCR-dependent T cell development

The vast majority of DP thymocytes die by neglect due to failure of their TCRs to interact with self-pMHC; the fate of the remainder is determined by the affinity of this interaction. DP thymocytes that experience low affinity TCR stimulation undergo positive selection, receiving cues for survival, migration from the cortex to the medulla, and differentiation into CD4 or CD8 single positive (SP) thymocytes (**Figure 1**). In contrast, high affinity TCR-pMHC interactions result in negative selection, which is primarily mediated by clonal deletion of thymocytes expressing the high affinity TCR. Low affinity TCR ligands are thought to be non-cognate self-peptides, whereas cognate antigen provides high affinity stimulation (Starr et al. 2003). During migration through the thymic cortex, DP thymocytes may receive both low and high affinity TCR signals, but negative selection is dominant over positive selection. These processes are strictly controlled and dysregulation can lead to the development of immunodeficiency and autoimmune disorders. The remainder of this chapter discusses TCR-induced signaling pathways during negative and positive selection and subsequent regulation of pro- and anti-apoptotic factors.

3.1. TCR signaling pathways in positive and negative selection

An important unresolved question in T cell development is how high and low affinity TCR stimulation is translated into negative and positive selection outcomes. The current model is centered on differential activation of the mitogen-activated protein kinases (MAPKs) ERK1/2, ERK5, p38, and c-Jun N-terminal kinase (JNK) (**Figure 2**). MAPK signaling cascades, which involve activation of a series of kinases (MEKK→MEK→MAPK), mediate responses to extracellular stimuli including TCR stimulation. ERK1/2 is known to promote survival and differentiation while JNK and p38 are linked to apoptosis in other systems (Xia et al. 1995). Several lines of evidence suggest that they have similar functions during thymocyte development. For example, deletion of JNK1 or JNK2 renders thymocytes more resistant to death upon anti-CD3 ϵ stimulation (Sabapathy et al. 1999; Sabapathy et al. 2001). Consistent with this, inhibition of JNK activity by a dominant-negative mutant inhibits peptide-induced negative selection *in vivo* (Rincon et al. 1998). Likewise, addition of a p38 inhibitor to a TCR transgenic fetal thymic organ culture impairs peptide-induced deletion (Sugawara et al. 1998). Characterization of the MEK5-ERK5 pathway is relatively recent compared to other MAPK signaling cascades. Dominant-negative ERK5 and MEK5 inhibit thymocyte apoptosis *in vitro* and peptide-induced deletion in some models of negative selection, respectively (Fujii et al. 2008; Sohn et al. 2008). While JNK, p38, and ERK5 have been implicated in negative selection, they are not required for positive selection of thymocytes (Rincon et al. 1998; Sugawara et al. 1998; Sohn et al. 2008). Conversely, inhibition of ERK1/2 or their activator MEK blocks positive selection but does not affect negative selection (Alberola-Ila et al. 1995; Alberola-Ila et al. 1996; Sugawara et al. 1998; Pages et al. 1999).

The upstream molecules that link high and low affinity TCR stimulation to differential MAPK activation are not well understood. Whereas premature TCR α expression inhibits β -selection, the TCR α chain is essential for positive selection of DP thymocytes (Mombaerts et al. 1992; Takahama et al. 1992; Lacorazza et al. 2001). This is because pMHC-induced signals impair development at the DN stage, whereas selection of DP thymocytes is dependent on pMHC ligands. It has been shown that CD3 δ is required to transduce positive selection but not β -selection signals (Dave et al. 1997). Interestingly, the TCR α chain contains a motif important for both peptide contact and retention of CD3 δ in the TCR complex, suggesting that CD3 δ is a critical link between ligand binding and signal transduction (Backstrom et al. 1998). Mutating this motif in the TCR α chain abrogates CD3 δ association with the TCR and ERK1/2 activation, resulting in defective peptide-induced positive selection (Werlen et al. 2000). This mutation has no effect on p38 and JNK activation or negative selection in the same system. During negative selection, JNK activation may be connected to the TCR complex through an upstream kinase called misshapen/NIKs-related kinase (MINK). This is evidenced by an association between CD3 ϵ , MINK, and the adaptor protein non-catalytic region of tyrosine kinase (Nck) after stimulation of TCR transgenic thymocytes with cognate peptide (McCarty et al. 2005) (**Figure 2**). Consistent with its role in activating JNK, inhibition of MINK activity impairs negative selection *in vivo*. Involvement of different CD3 chains may result in differential phosphorylation of linker for activation of T cells (LAT), the

central adaptor protein that links TCR proximal and distal signaling pathways (Starr et al. 2003). For example, regulation of different MAPK pathways through the adaptor protein growth factor receptor-bound protein 2 (Grb2) and the Ras activating protein Ras guanyl-releasing protein 1 (RasGRP1) in TCR signaling is thought to result from phosphorylation of different LAT residues (Wange 2000). Grb2 was shown to be important for p38 and JNK activation and negative selection, whereas RasGRP1 is essential for ERK1/2 activation and positive selection (Dower et al. 2000; Gong et al. 2001). However, a recent study reported that Grb2^{-/-} mice are impaired in both negative and positive selection (Jang et al. 2010). Though much remains unknown about the discrimination of positive and negative selection signals, these findings shed light on how DP thymocyte fate is determined by TCR-pMHC interactions.

Figure 2. Signaling pathways in positive and negative selection.

Selection of DP thymocytes depends on the affinity of the TCR for self-pMHC in the thymus. Low affinity TCR-pMHC interactions result in positive selection and high affinity interactions in negative selection. The TCR is composed of one α and β chain each and transduces signals through a complex consisting of CD3 chains (γ, δ, ε, ζ). TCR proximal

signaling events involve activation of kinases Lck and Zap70. Differential phosphorylation of the adaptor protein LAT is thought to result in activation of different MAPK pathways. The MAPKs JNK and p38 are important for negative selection and ERK1/2 for positive selection. JNK activation has also been linked to the TCR complex through the adaptor protein Nck and kinase MINK. ERK5 is another, relatively uncharacterized MAPK that may contribute to negative selection (not depicted). Active MAPK pathways are thought to lead to induction of pro-survival and pro-death factors that mediate positive or negative selection. (Lck - lymphocyte-specific protein tyrosine kinase; Zap70 - zeta chain-associated protein kinase 70.)

3.2. Induction of survival factors during positive selection

TCR signaling during positive selection results in reacquisition of IL-7 responsiveness in post-selection DP and SP thymocytes (Van De Wiele et al. 2004; Marino et al. 2010). Neutralizing IL-7R α has been shown to inhibit SP development upon transfer of thymocytes from a non-selection (MHC^{-/-}) to a positive selection (MHC^{+/+}) background (Akashi et al. 1997). However, IL-7R α ^{-/-} mice have a normal frequency of SP thymocytes (Peschon et al. 1994). Characterization of the role of IL-7 in positive selection may be confounded by the requirement for IL-7 in DN survival and proliferation. While IL-7 may not be required for positive selection, it is thought that IL-7 signaling provides important survival cues in SP thymocytes. Along with IL-7 responsiveness, Bcl-2 and Mcl-1 expression are upregulated in SP thymocytes (Linette et al. 1994; Akashi et al. 1997; Marino et al. 2010). Since Mcl-1 regulates early DN survival, a CD4-cre recombinase system was used to conditionally delete Mcl-1 at the DP stage. Positive selection is impaired in the absence of Mcl-1, as indicated by a reduced number of SP thymocytes (Dzhagalov et al. 2008; Dunkle et al. 2010). Mcl-1 deficiency is partially rescued by transgenic Bcl-2 expression, suggesting that Mcl-1 and Bcl-2 act on overlapping and distinct targets (Dunkle et al. 2010). Likewise, Bcl-2^{-/-} mice exhibit a partial decrease in SP numbers, consistent with the idea of other proteins providing redundant and non-redundant functions during positive selection (Wojciechowski et al. 2007). Bcl-2^{-/-} thymi are marked by a high frequency of DNA fragmentation, as indicated by positive staining in the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, as well as loss of thymocytes in the medulla, where SP cells normally reside (Veis et al. 1993). Conversely, transgenic Bcl-2 expression enhances positive selection and even allows SP development in the absence of MHC, though additional pMHC-induced signals are required for full maturation (Linette et al. 1994; Williams et al. 1998). The transcription factor c-Fos has been identified as an activator of Bcl-2 expression and also promotes positive selection on polyclonal and transgenic TCR backgrounds (Wang et al. 2009). Interestingly, c-Fos is implicated as a sensor for ERK1/2 signal duration (Murphy et al. 2002). Therefore, sustained ERK1/2 signaling during positive selection may be translated into c-Fos stabilization and Bcl-2 induction. Despite promoting thymocyte survival during positive selection, Bcl-2 is limited in its ability to inhibit clonal deletion of autoreactive thymocytes during negative selection (Sentman et al. 1991; Strasser et al. 1991).

3.3. The role of intrinsic and extrinsic apoptosis pathways in clonal deletion

Clonal deletion is widely held to occur through apoptosis, which can be mediated by extrinsic and intrinsic pathways. Many studies have examined the role of death receptors in negative selection. Mice defective for Fas or Fas ligand have been shown to have normal deletion of autoreactive thymocytes in multiple TCR transgenic models (Sidman et al. 1992; Singer and Abbas 1994; Sytwu et al. 1996). Similarly, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to have no effect on peptide- or superantigen-induced clonal deletion (Simon et al. 2001; Cretney et al. 2003; Cretney et al. 2008). However, other studies report that Fas and TRAIL signaling do contribute to negative selection under some conditions (Kishimoto et al. 1998; Lamhamedi-Cherradi et al. 2003). Many of these studies utilize models of negative selection in which exogenous antigen is injected *in vivo*. This can cause non-specific thymocyte deletion by the secreted products of activated peripheral T cells (Martin and Bevan 1997). Because activation-induced cell death of peripheral T cells is impaired in the absence of death receptors, results derived from these model systems may be complicated by involvement of extrathymic factors (Singer and Abbas 1994; Sytwu et al. 1996). Importantly, transgenic expression of dominant-negative FADD or the viral caspase-8 inhibitor cytokine response modifier A (CrmA) does not impair negative selection, strongly suggesting that the extrinsic apoptosis pathway is dispensable (Smith et al. 1996; Walsh et al. 1998; Newton et al. 2000).

As discussed in detail in the following section, there is strong evidence that the intrinsic apoptosis pathway is involved in negative selection. Caspase-3 is widely held to be the main executioner caspase in mammals since both death receptor and mitochondrial-initiated pathways converge on its activation. Activation of caspase-3 is observed in thymocytes stimulated *in vitro* and from TCR transgenic models of negative selection (Alam et al. 1997; Hu et al. 2009). In contrast, the active forms of executioner caspases-6 and -7 are not detected after stimulation of TCR transgenic thymocytes with cognate peptide but can be induced by non-specific stimulation with staurosporine (Hara et al. 2002). Furthermore, comparison of caspase-3^{-/-}, caspase-7^{-/-}, and double knockout thymocytes indicates that caspase-3 is mainly responsible for DNA fragmentation in response to anti-CD3 ϵ and anti-CD28 stimulation (Lakhani et al. 2006). Taken together, these data suggest that caspase-3 is the primary executioner caspase in clonal deletion. Though it normally plays a major role in clonal deletion, caspase-3 activation is not strictly required for negative selection *in vivo* (Hu et al. 2009; Murakami et al. 2010). While a role for other executioner caspases in negative selection has not been excluded, numerous studies using pan-caspase inhibitors *in vitro* and *in vivo* have shown that TCR-induced thymocyte death can occur in the absence of caspase activity, albeit to reduced levels in some systems (Alam et al. 1997; Izquierdo et al. 1999; Doerfler et al. 2000; Hara et al. 2002). These data highlight the existence of multiple mechanisms that have evolved to mediate negative selection and self-tolerance.

3.4. Initiators of the intrinsic apoptosis pathway

Mitochondria are central to the initiation of caspase-dependent and caspase-independent cell death (Jaattela and Tschopp 2003). Of the Bcl-2 family that controls the mitochondrial

gateway to cell death, the pro-apoptotic protein Bim has a critical role in TCR-induced thymocyte death. Bim induction has been demonstrated after TCR stimulation of thymocytes *in vitro* and in numerous models of negative selection *in vivo* (Bouillet et al. 2002; Schmitz et al. 2003; Huang et al. 2004; Zucchelli et al. 2005; Baldwin and Hogquist 2007; Liston et al. 2007). The JNK pathway positively regulates Bim expression and function in other cell types (Whitfield et al. 2001; Putcha et al. 2003). Post-translational modification does not seem to be a major mechanism by which the TCR regulates Bim in thymocytes (Bunin et al. 2005). In thymocytes, MINK activity is linked to JNK activation and Bim induction, though it was not shown that Bim induction is JNK-dependent (McCarty et al. 2005). Another study found that PKC inhibitors, but not JNK, p38, or MEK inhibitors, block Bim induction in thymocytes stimulated with anti-CD3 ϵ and anti-CD28 (Cante-Barrett et al. 2006). However, unlike JNK and p38, PKC activity is not required for negative selection (Anderson et al. 1995; Sun et al. 2000). More work is required to clarify the pathways activated during negative selection that lead to regulation of Bim.

Bim is thought to have a critical role in clonal deletion as Bim deficiency results in resistance to TCR-induced thymocyte apoptosis *in vitro* (Bouillet et al. 2002) and abrogation of caspase-3 activation in DP thymocytes undergoing negative selection *in vivo* (Hu et al. 2009). Consistent with its essential role in thymocyte apoptosis, deletion of DP thymocytes by superantigen and cognate peptide is impaired in the absence of Bim (Bouillet et al. 2002). Though deletion of DP thymocytes is indicative of clonal deletion, the number of autoreactive SP thymocytes is the most accurate measure of negative selection. In the physiological HY^{cd4} model of negative selection, Bim deficiency delays deletion of DP thymocytes but the number of autoreactive SP thymocytes is ultimately comparable in the presence and absence of Bim (Hu et al. 2009). Because Bim is required for caspase-3 activation in this model, these data support the idea of a redundant non-apoptotic cell death mechanism of negative selection. In addition, superantigen-mediated negative selection, as measured by autoreactive SP numbers, has also been reported to be Bim-independent (Jorgensen et al. 2007).

Another factor to consider when evaluating the role of a protein in negative selection is the model system utilized. The molecular events involved in superantigen-induced deletion may be different from those initiated from pMHC interactions. Mice expressing transgenic TCRs against endogenous or neo self-antigens (thus avoiding the issues associated with peripheral T cell activation) have been the most powerful tools available for studying negative selection. However, there are key differences between TCR transgenic models. For example, in the classical HY model where HY is a male-specific antigen, the transgenic HY TCR is prematurely expressed on DN thymocytes such that deletion occurs during the DN to DP transition (Takahama et al. 1992; Baldwin et al. 2005). Thus, while Bim deficiency impairs negative selection in the HY model, this may in part reflect a role for Bim in DN thymocyte death (Bouillet et al. 2002). The HY^{cd4} model utilizes a CD4-cre recombinase system to conditionally express the transgenic HY TCR α chain at the DP stage, allowing selection to occur during the DP to SP transition as in wildtype mice (Baldwin et al. 2005). When the timing of TCR expression has been corrected, Bim appears to be dispensable for deletion of HY TCR⁺ thymocytes (Hu et al. 2009; Kovalovsky et al. 2010).

The stage of development at which deletion occurs has considerable implications on the molecular mechanism involved due to each thymocyte subset having differential gene expression, signaling threshold, and localization. For example, positive selection induces differentiation of DP into SP and migration to the medulla. Localization affects antigen presentation because the thymic cortex and medulla contain different types of antigen presenting cells. Importantly, ectopic expression of tissue-restricted antigens such as insulin is restricted to medullary thymic epithelial cells (Derbinski et al. 2001; Anderson et al. 2002). Because HY is a ubiquitous antigen, clonal deletion of HY^{cd4} thymocytes occurs at the DP stage in the cortex, a process that does not require Bim (McCaughy et al. 2008; Hu et al. 2009). While past studies have cited defective Bim induction and clonal deletion in type I diabetes-prone non-obese diabetic (NOD) mice (Zucchelli et al. 2005; Liston et al. 2007), a recent study clarified that NOD mice do not have a cell-intrinsic impairment in clonal deletion (Mingueneau et al. 2012). Negative selection against tissue-restricted antigens was also recently examined by transfer of OT-I or OT-I Bim^{-/-} TCR transgenic bone marrow, which specifically recognizes ovalbumin peptide, into recipients in which the cognate antigen is driven by the ubiquitously active actin promoter or the tissue-restricted rat insulin promoter (Suen and Baldwin 2012). In agreement with results from the HY^{cd4} model, Bim is not required for negative selection against ubiquitous antigen, but is required for negative selection against tissue-restricted antigen in a cell-intrinsic way (**Table 1**). Because DP thymocytes are more sensitive to TCR-induced death than SP thymocytes (Davey et al. 1998), one explanation is that either a Bim-dependent or independent mechanism is sufficient to kill DP cells, while both are required for SP deletion. Alternatively, interactions with medullary thymic epithelial cells may not induce factors that mediate Bim-independent cell death.

The Bcl-2 family are critical regulators of mitochondrial integrity. The balance of pro-apoptotic and anti-apoptotic members controls the activation of Bax and Bak, which leads to cytochrome c release and caspase activation. Programmed cell death plays an important role in the elimination of dysfunctional and autoreactive thymocytes. This table summarizes some of the proteins known to play a role in thymocyte survival. Different Bcl-2 members are important at different stages of thymocyte development.

One candidate for mediating Bim-independent clonal deletion is the NR4A nuclear receptor Nur77. The NR4A nuclear receptor family is comprised of three proteins closely related in structure and function: Nur77, Nor-1, and Nurr1, though only Nur77 and Nor-1 are induced in stimulated thymocytes (Cheng et al. 1997). Nur77 is induced by TCR stimulation of thymocytes and DO11.10 T cell hybridoma cells *in vitro* (Liu et al. 1994; Woronicz et al. 1994) and is consistently among the list of genes upregulated during negative selection *in vivo* (Schmitz et al. 2003; Huang et al. 2004; Zucchelli et al. 2005; Baldwin and Hogquist 2007; Liston et al. 2007). Deletion of the transactivation domain of Nur77 creates a dominant-negative mutant that interferes with the transcriptional activity of all NR4A family members (Cheng et al. 1997). Expression of dominant-negative Nur77 partially impairs clonal deletion in some TCR transgenic models but not others (Calnan et al. 1995; Zhou et al. 1996). Past studies with Nur77^{-/-} mice reported normal negative selection *in vivo*, suggesting that Nor-1

provides redundant functions in thymocytes (Lee et al. 1995). Nevertheless, little is known about Nur77 compared to Nur77. Recently, Nur77 deficiency alone has also been shown to impair negative selection (Fassett et al. 2012). The MEK5-ERK5 pathway has been reported to induce Nur77 expression in thymocytes and DO11.10 cells (Kasler et al. 2000; Sohn et al. 2008). However, MEK5 and ERK5 have not been shown to be required for Nur77 induction in response to TCR stimulation. Indeed, ERK5 is not necessary for Nur77 induction upon activation of peripheral T cells (Ananieva et al. 2008). Consistent with dominant-negative Nur77 studies, expression of dominant-negative MEK5 inhibits clonal deletion in certain TCR transgenic models (Sohn et al. 2008). Dominant-negative ERK5 has been shown to inhibit apoptosis in DO11.10 cells *in vitro* but characterization of ERK5 in negative selection *in vivo* is presently limited. Because pharmacological inhibitors can act on both MEK1/2 (upstream of ERK1/2) and MEK5 at high doses (Mody et al. 2001), it is possible that MEK5 can act on additional targets other than ERK5. Taken together, these data support a role for Nur77 in some types of negative selection.

	Pre- β -selection	β -selection	Death by neglect	TCR-dependent selection
Anti-apoptotic members				
Bcl-2	✓			✓
Mcl-1	✓		✓	✓
Bcl2A1		✓		
Bcl-x _L			✓	
Pro-apoptotic members				
Bad	✓			
Bim		✓	✓	✓
Bid		✓		
Puma			✓	

Table 1. Regulation of survival by the Bcl-2 family during thymocyte development.

The mechanism of Nur77-induced thymocyte death is controversial. Nur77 was initially thought to upregulate factors that mediate apoptosis since the transcriptional activity of Nur77 correlates with its ability to induce thymocyte death (Kuang et al. 1999). Furthermore, Nur77 has been shown to remain in the nucleus of stimulated thymocytes, while nuclear export of Nur77 in mature T cells is thought to protect them from apoptosis (Cunningham et al. 2006). However, the only target genes of Nur77 with known apoptotic function are those

involved in the extrinsic apoptosis pathway (Rajpal et al. 2003). Thus, the role of Nur77-mediated transcription in negative selection has been questioned. Studies of Nur77 function in cancer cells have identified a mitochondrial, transcription-independent cell death mechanism whereby Nur77 converts Bcl-2 into a pro-apoptotic protein by exposing its Bcl-2 homology 3 (BH3) domain (Lin et al. 2004). By utilizing a subcellular fractionation protocol not used in previous studies, Nur77 was reported to translocate to mitochondria and associate with Bcl-2 upon TCR stimulation of thymocytes (Thompson and Winoto 2008). While other groups also report mitochondrial translocation of Nur77 following stimulation (Stasik et al. 2007; Wang et al. 2009), whether Nur77 mediates thymocyte death through Bcl-2 conversion is controversial. In stimulated DO11.10 cells, Bcl-2 and Nur77 do not interact and Bcl-2 expression protects against Nur77-mediated death (Wang et al. 2009). Furthermore, Bcl-x_L, not Bcl-2, is the predominant survival factor expressed in DP thymocytes; transgenic overexpression of Bcl-2 was required to detect interaction with Nur77 (Ma et al. 1995; Thompson and Winoto 2008). Studies also differ on regulation of Nur77 nuclear export. In DO11.10 cells, phosphorylation of serine 354 by the ERK1/2-ribosomal S6 kinase (RSK) pathway is necessary for nuclear export (Wang et al. 2009). This is contested by another study that found PKC but not ERK1/2 to be required for mitochondrial translocation (Thompson et al. 2010). Since ERK1/2 (Alberola-Ila et al. 1995; Alberola-Ila et al. 1996; Sugawara et al. 1998; Pages et al. 1999) and PKC (Anderson et al. 1995; Sun et al. 2000) are not required for negative selection, the contribution of the transcription-independent mechanism of Nur77 to clonal deletion is also questionable.

Mice that express transgenic Nur77 or transgenic Nor-1 have severely reduced thymic cellularity and an increased frequency of TUNEL⁺ thymocytes (Calnan et al. 1995; Cheng et al. 1997). Conversely, thymocytes from DN-Nur77 mice are more resistant to DNA fragmentation following anti-CD3 ϵ stimulation (Zhou et al. 1996). Surprisingly, transgenic Nur77 expression does not appear to be sufficient to induce cytochrome c release (Rajpal et al. 2003). A caveat of using these transgenic mice to study negative selection is that Nur77 transgene expression is driven by promoters that are active in DN thymocytes independently of TCR signaling. Thus, transgenic Nur77 may induce thymocyte death through transcription of extrinsic apoptosis genes, while physiological regulation of Nur77 by TCR signaling may favor a transcription-independent mechanism. Studies using T cell hybridomas generally support activation of the intrinsic apoptosis pathway by Nur77. For example, nuclear export of Nur77 in DO11.10 cells leads to cytochrome c release, caspase-9 cleavage, and poly(ADP-ribose) polymerase (PARP) cleavage (Wang et al. 2009). Though T cell hybridomas are more amenable to manipulation than thymocytes, it is important to keep in mind that the mechanism of Nur77-mediated death may differ between cell types. Despite induction of apoptosis, z-VAD treatment and Bcl-2 or Bcl-X_L expression only partially rescues Nur77-induced cell death, suggesting contribution of a caspase-independent mechanism. Interestingly, Nur77 has been shown to mediate caspase-independent death in other cell types (Kim et al. 2003; Castro-Obregon et al. 2004; Lucattelli et al. 2006). Furthermore, it is unknown whether the DNA fragmentation induced by Nur77 in thymocytes is caspase-dependent oligonucleosomal fragmentation or caspase-independent large scale DNA fragmentation since the TUNEL assay does not discriminate between the two types (Ribeiro et al. 2006).

Abbreviation	Full name	Description
AIF	Apoptosis-inducing factor	Protein released from mitochondria that mediates cell death
AMPK	ADP-activated protein kinase	Regulates cellular energy metabolism in response to ATP depletion
Apaf-1	Apoptotic protease-activating factor 1	Activates caspase-9 when bound to cytochrome c and dATP
Bcl-2	B cell lymphoma 2	Founding, anti-apoptotic member of the Bcl-2 family of proteins, initially described in B cell lymphomas. Bcl-xL and Bcl2A1 are similarly named.
Bad	Bcl-2-associated death promoter	Pro-apoptotic member of the Bcl-2 family
Bak	Bcl-2 homologous antagonist/killer	Pro-apoptotic member of the Bcl-2 family that forms channels in the mitochondrial outer membrane
Bax	Bcl-2-associated X protein	Pro-apoptotic member of the Bcl-2 family that forms channels in the mitochondrial outer membrane
Bid	Bcl-2 homology 3 interacting-domain death agonist	Pro-apoptotic member of the Bcl-2 family
Bim	Bcl-2-interacting mediator of cell death	Pro-apoptotic member of the Bcl-2 family
CXCR4	C-X-C motif receptor 4	Chemokine receptor
DN	Double negative	CD4 ⁻ CD8 ⁻ thymocyte subset
DP	Double positive	CD4 ⁺ CD8 ⁺ thymocyte subset
ERK	Extracellular signal-regulated kinase	A subfamily of mitogen-activated protein kinases
FADD	Fas-associated death domain	Adaptor protein involved in signaling through death receptors
Grb2	Growth factor receptor-bound protein 2	Adaptor protein involved in T cell receptor signaling
JNK	c-Jun N-terminal kinase	A subfamily of mitogen-activated protein kinases
MAPK	Mitogen activated protein kinase	A class of kinases that respond to extracellular stimuli
Mcl-1	Myeloid leukemia sequence 1	Anti-apoptotic member of the Bcl-2 family
MHC	Major histocompatibility complex	Protein that presents peptide antigen to T cell receptor

MINK	Misshapen/NIKs-related kinase	Kinase involved in T cell receptor signaling that promotes JNK activation
Nck	Non-catalytic region of tyrosine kinase	Adaptor protein involved in T cell receptor signaling
NOD	Non-obese diabetic	Mouse strain genetically predisposed to developing type I diabetes
PI3K	Phosphoinositide 3-kinase	Phosphorylates phosphatidylinositol (4,5)-biphosphate to generate phosphatidylinositol (3,4,5)-triphosphate, leading to Akt activation
PKC	Protein kinase C	Kinase involved in T cell receptor signaling
pMHC	Peptide-MHC	Peptide presented on an MHC molecule
PTEN	Phosphatase and tensin homolog	Dephosphorylates phosphatidylinositol (3,4,5)-triphosphate to generate phosphatidylinositol (4,5)-biphosphate, negatively regulating Akt activation
Puma	p53-upregulated modulator of apoptosis	Pro-apoptotic member of the Bcl-2 family
Rag	Recombination activating gene	Enzyme that mediates genetic recombination of T cell receptor loci
RasGRP1	Ras-guanyl releasing protein 1	Activates Ras through the exchange of bound GDP for GTP
RBP-J	Recombination signal binding protein for immunoglobulin kappa J region	Transcriptional regulator that activates transcription when bound to intracellular domain of Notch proteins
RORγt	Retinoic acid-related orphan receptor γ t	Transcription factor that induces Bcl-xL expression in thymocytes
SP	Single positive	CD4 ⁺ CD8 ⁻ or CD4 ⁺ CD8 ⁺ thymocyte subsets
TCR	T cell receptor	Antigen receptor expressed by T cells
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand	Protein that induces apoptosis by binding to death receptors
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling	Method of detecting DNA fragmentation by labeling the terminal end of fragments

Table 2. Abbreviations used multiple times throughout this chapter.

The molecular mechanisms of negative selection remain unclear despite intense investigation into the matter. Though Bim and Nur77 are implicated as key mediators of TCR-induced thymocyte death, many questions surround their role in physiological negative selection. Consideration of the model system and readout to be used will greatly assist future endeavors to clarify the molecular mechanisms of negative selection.

4. Conclusion

The highly regulated struggle between survival and death during thymocyte development underscores the need to generate functional yet self-tolerant T cells. At multiple checkpoints during development in the thymus, the balance must be tipped in favour of pro-death factors to allow elimination of dysfunctional and autoreactive cells. It is becoming increasingly apparent that programmed cell death is not restricted to apoptosis but also involves caspase-independent processes such as autophagy and necroptosis. While non-apoptotic programmed cell death is better characterized in peripheral T cells (Jaattela and Tschopp 2003), many studies have provided evidence that caspase-independent death can occur at multiple stages of thymocyte development. Two proteins implicated in caspase-independent death are apoptosis-inducing factor (AIF) and endonuclease G. Both have been shown to mediate cell death following translocation from mitochondria to the nucleus, where they execute caspase-independent DNA fragmentation (Jaattela and Tschopp 2003). It will be of great interest to determine if AIF and endonuclease G contribute to caspase-independent thymocyte death, and if their translocation is induced by Nur77, which has been linked to mitochondrial localization and caspase-independent cell death. One thing is clear: mitochondria are a key gateway to cell death. The Bcl-2 family of proteins control mitochondrial integrity through regulating formation of Bax/Bak channels and opening of the mitochondrial permeability transition pore (Tsujimoto and Shimizu 2000). Therefore, they may be poised to control different mechanisms of programmed cell death. Though genetic manipulation of mice has accelerated our understanding of thymocyte survival and death, much remains to be characterized about the molecular mechanisms involved. These complex, interwoven, and tightly regulated mechanisms are necessary to balance the need for a competent and self-tolerant immune system.

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5. References

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