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Negative selection and autoimmunity

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The impaired elimination of self-reactive T cells is one factor that contributes to autoimmunity. Although the mechanism of thymic negative selection has been studied for decades, recent data demonstrate that the mechanisms underlying this fundamental process remain extremely controversial. Nonetheless, new models such as the Aire-deficient mice have demonstrated the importance of thymic negative selection in autoimmune disease progression in mice and humans.

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Abbreviations

Aire	autoimmune regulator
DC	dendritic cell
DN	CD4 [−] CD8 [−] double negative
DP	CD4 ⁺ CD8 ⁺ double positive
DR	death receptor
ERK	extracellular signal-regulated kinase
HEL	hen egg lysozyme
IL	interleukin
IκB	inhibitor of κB
JNK	jun amino-terminal kinase
MAP	mitogen-activated protein
MKK	MAP kinase kinase
MEC	medullary epithelial cell
NF-κB	nuclear factor κB
PI3K	phosphatidylinositol-3 kinase
PKB	protein kinase B
SAPK	stress-activated protein kinase
SP	CD4 ⁺ or CD8 ⁺ single positive
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF-related apoptosis-inducing ligand

Introduction

In the thymus, negative selection is an important process that functionally eliminates self-reactive T cells. Two major mechanisms have been proposed to trigger the negative selection of thymocytes. One model proposes that T-cell death is triggered by members of the death receptor TNF/TNF receptor (TNFR) family (Figure 1). The second model suggests that the differential activation of signaling pathways is a key process triggering the

expression of genes that regulate differentiation, survival or apoptosis. Evidence suggests that the extracellular signal-regulated kinase (ERK) pathway signals positive selection, whereas the activation of the jun amino-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 pathway lead to negative selection (Figure 2; reviewed in [1,2]).

In this review, the term ‘apoptosis’ is not interchangeable with ‘negative selection’. I refer to apoptosis as the process of death that is balanced by survival signals to maintain thymocyte homeostasis and is inherently programmed during the different stages of thymocyte development. Apoptosis is influenced by cytokines and glucocorticoids. Negative selection is death that is induced after the high affinity/avidity interactions between self-antigens and specific T-cell receptors (TCRs) expressed by developing thymocytes. Clearly, negative selection is ultimately linked with death. However, the mechanism of negative selection should not be confused with, and does not necessarily have to be linked with, thymocyte homeostasis.

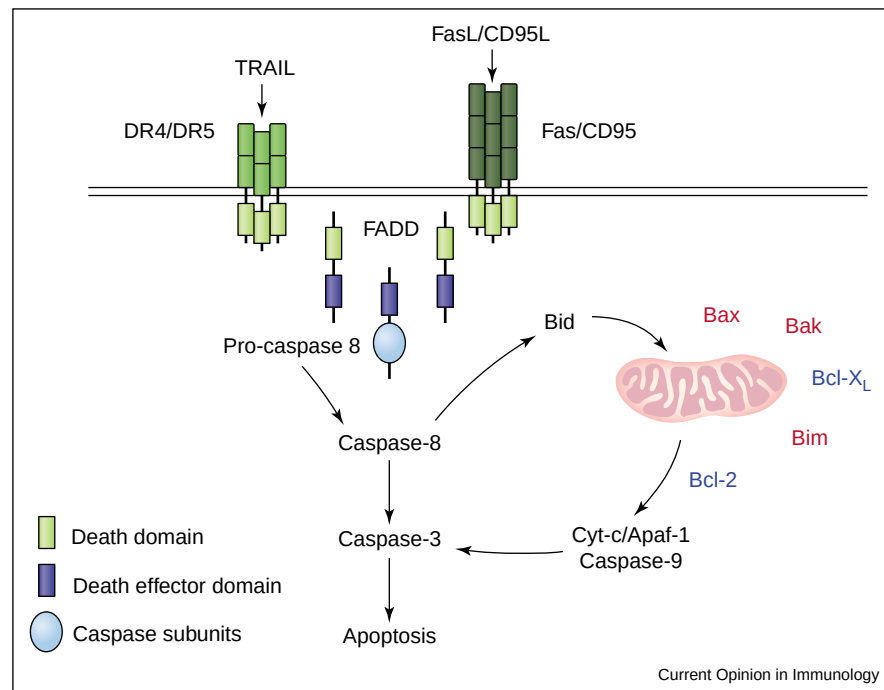
This review focuses on negative thymocyte selection and the recent advances that address the mechanisms of negative selection. The link between impaired negative selection and autoimmunity in relevant animal models is also discussed.

Negative selection: models, predictions and restrictions

The deletion of self-reactive thymocytes may occur either at the CD4⁺CD8⁺ double positive (DP) stage of development, or at the more mature CD4⁺ or CD8⁺ single positive (SP) stage (Figure 3; [3–5]). The timing of deletion is dependent upon the model self-antigen, the affinity/avidity of the self-reactive T cell for the self-antigen, and the location of the cells that express the self-antigen [6–8]. Many transgenic models have shown that deletion may occur at the DP stage of thymocyte development, many researchers still believe that deletion to physiological self-antigens occurs later in the medulla. Although this has not been unequivocally proven, the fact that deletion can occur earlier suggests that the machinery and mechanisms that dictate positive versus negative selection can work at the earlier DP stage.

The timing of deletion has important implications for the underlying mechanisms of negative selection. Many studies have suggested that certain genes are involved in negative selection by demonstrating that deleting or blocking signaling molecules inhibits negative selection.

Figure 1



The induction of thymocyte negative selection by death receptors. One model for the mechanism of negative selection suggests that autoreactive thymocytes die by engaging death receptors and appropriate ligands. Fas/Fas ligand, TRAIL/DR4/DR5 and the shared downstream molecules involved in the induction of apoptosis are shown. Pro-apoptotic molecules are shown in red (Bax, Bak and Bim) and pro-survival molecules are shown in blue (Bcl-2, Bcl-X_L). Other death-domain-containing proteins of the TNFR superfamily that are not shown are TNFR1, DR3 and DR6.

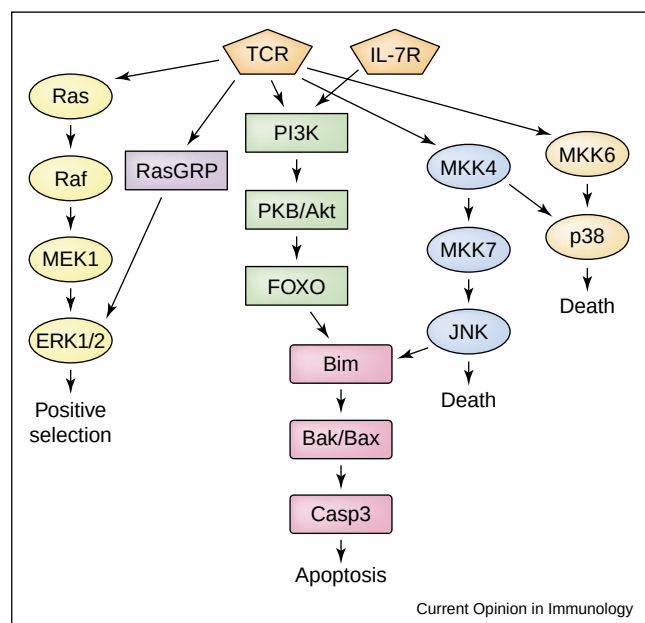
If negative selection is blocked at the DP stage, then one prediction would be that T cells could still edit the TCR, because the DP thymocytes may still be programmed to survive for several days (Figure 3, red arrows; [9,10]). As a consequence of editing, another prediction would be that many thymocytes will be able to undergo positive selection and the SP populations would appear expressing alternative TCRs. However, if the self-antigen is found in the medulla, or if the affinity is such that the increase in TCR density that occurs during selection is required to achieve the signals for deletion, then blocking the deletion of the self-reactive T cell would not allow subsequent receptor editing and positive selection (Figure 3, red arrows). Therefore, depending on the model, the role of any given molecule in negative selection would lead to the presence or absence of different thymocyte populations.

Many studies that examine mechanisms of negative selection have used the H-Y transgenic model, which expresses a TCR specific for the H-Y male antigen and H-2D^b. Blocking negative selection at the double positive stage should restore normal thymic cellularity and lead to the maturation of T cells expressing an alternative TCR (a shift from negative to positive selection). Thus, one would expect to see the presence of both CD4⁺ and

CD8⁺ SP subsets in the thymus and periphery, and not just a partial 'rescue' of the DP population, as reported in many studies. In fact, studies have shown that it is possible to rescue thymic cellularity and the DP population during negative selection in the H-Y transgenic model [11], although this is generally not achieved in most studies.

One major complication in studying the mechanisms of negative selection of DP thymocytes is the fact that survival of DP thymocytes is influenced by multiple pathways, including cytokines ([12,13]; and reviewed in [14]). It is clear that IL-7 plays an important role in thymocyte survival. The transition from the immature CD4⁺CD8⁺ double negative (DN) stage to the CD4⁺CD8⁺ DP stage can occur by promoting survival (via Bcl-2) in the absence of IL-7 ([15,16]; see also Update). Recent studies have also shown that IL-7 plays an important role in the survival and differentiation of DP thymocytes into mature CD8⁺ cells [17]. Many studies examining whether molecules block negative selection interpret the recovery of DP cells as a sign that negative selection has been rescued. An alternative interpretation, however, is that the experiments may influence DP apoptosis, rather than the process of negative selection itself.

Figure 2



Pathways related to selection. Positive selection is triggered by activating the ERK pathway. Negative selection has been associated with the induction of JNK/SAPK and p38. The kinetics of activation of these pathways, and possibly the degree of induction, discriminate between signals for positive or negative selection [1,2]. Ovals represent molecules involved in MAP kinase pathways. Rectangles represent molecules triggered by phospholipid metabolism. Rounded rectangles are molecules involved in regulating apoptosis. MEK, MAP kinase extracellular signal-regulated kinase; MKK, MAP kinase kinase; RasGRP, Ras guanyl nucleotide releasing protein.

Another complication to understanding the mechanisms of negative selection is the number of different models that are used. Many reports in the literature use anti-CD3 treatment *in vivo* to induce thymocyte death. This is then directly translated to address mechanisms of negative selection. However, anti-CD3 treatment induces the activation of mature thymocytes and peripheral T cells. This promotes a corticosterone surge that favors the death of the glucocorticoid-sensitive DP population [18^{••}]. In addition, previous reports have shown that anti-CD3 treatment also causes the thymic stromal cells to upregulate a variety of cytokines [19]. Therefore, a multitude of events are triggered *in vivo* upon anti-CD3 treatment. The direct translation of any results from this type of experiment to the process of negative selection must clearly be made with caution. In many cases, these models are testing the 'stress' resistance of DP thymocytes, or the ability of the TCR to signal, rather than examining the process of negative selection.

Negative selection and autoimmunity is influenced by the transcription factor Aire

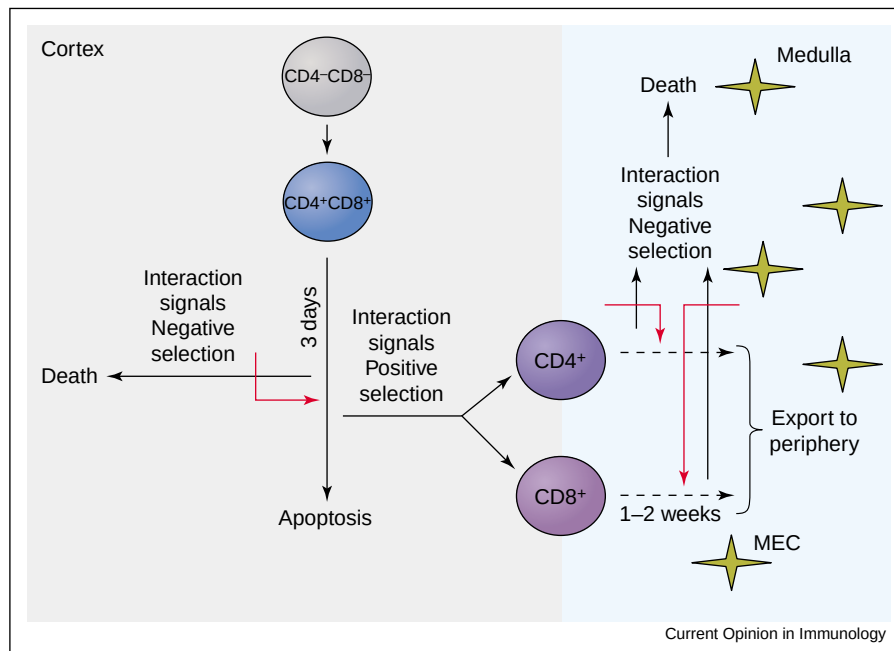
Various stromal cell populations that mediate the process of central tolerance have been examined. Studies suggest

that both bone-marrow-derived cells and radioresistant stromal cells can mediate this process [20,21]. A rare population of cells from the thymus called medullary epithelial cells (MECs) has also been examined. Although debated by some groups [22], studies have shown that these MECs have the unusual ability to express proteins that are generally expressed outside the thymus in a tissue-specific manner [23–25]. Recent studies suggest that this ectopic expression of tissue-specific proteins plays an important role in central tolerance induction and the prevention of autoimmunity.

Autoimmune regulator (AIRE) is a transcription factor that is mutated in a human disorder called autoimmune polyendocrinopathy-candidiasis ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome-type 1 (APS-1) [26,27]. In this disorder, autoimmunity to multiple organs is characteristically observed in endocrine organs, the skin and the gastrointestinal tract (reviewed in [28,29]). It has recently been reported that *Aire* gene-deficient animals have a similar phenotype to human APECED [30[•],31^{••}]. Gene expression profiles were examined in MECs from wild-type and *Aire*^{-/-} mice. Strikingly, many ectopically expressed tissue-specific genes were downregulated in the absence of Aire. This suggests that Aire may be a key transcriptional regulator of tissue-specific genes in the thymic MECs. In the absence of *Aire*, thymic tolerance should be defective, allowing self-reactive T cells to escape and contribute to the development of organ-specific autoimmunity.

Recent studies have directly examined whether thymic tolerance is impaired in the absence of *Aire*. To investigate T-cell tolerance to a defined self-antigen, a double transgenic model was used. The 3A9 transgenic model, expressing a TCR specific for hen egg lysozyme (HEL) and I-A^k was combined with InsHEL transgenic mice, which express HEL under the control of the rat insulin promoter [32[•]]. This leads to the expression of HEL in the β -islet cells of the pancreas. Lower concentrations of HEL are also found in the systemic circulation. Interestingly, the 3A9 TCR/InsHEL double transgenic mice show a defect in thymic deletion in the absence of *Aire*, when compared to *Aire*^{+/-} or *Aire*^{+/+} mice. This suggests that Aire contributes to thymic deletion. In this model, however, it is not clear whether the HEL antigen is expressed by MECs, although reports suggest that insulin can be produced in the MECs [23,31^{••}]. In addition, it is not clear whether the 3A9 TCR is normally positively selected in the absence of *Aire*. In chimera experiments, *Aire*^{-/-} mice reconstituted with bone marrow from 3A9 TCR transgenic *Aire*^{+/+} mice show a reduced proportion of CD4⁺ transgenic T cells compared to the number of CD4⁺ transgenic cells following the reconstitution of B10BR *Aire*^{+/+} mice (the control strain). In fact, antigen-expressing HEL/*Aire*^{-/-} mice reconstituted with bone marrow from 3A9 *Aire*^{+/+} donors show the same

Figure 3



Balancing thymocyte survival and apoptosis, positive and negative selection. Thymocyte development is a process that follows a predefined differentiation program. Studies have shown that DP cortical thymocytes survive for approximately three days before undergoing programmed cell death [95]. During this time, the thymocyte may undergo positive or negative selection depending upon the specificity of the rearranged TCR that is expressed on the surface. These processes are influenced by a variety of other surface receptors. Positively selected thymocytes that differentiate to the $CD4^+$ or $CD8^+$ SP stage may be negatively selected by MECs, DCs or other antigen-presenting cells found in the medulla. Manipulations that block negative selection have different predictions depending upon the negative selection model being studied. Blocking negative selection in the H-Y model would increase the survival of DP thymocytes and permit the positive selection of both SP subsets. Blocking negative selection to self-antigens expressed in the medulla should not have a striking effect on the DP subset.

reduced proportion of reconstituted 3A9 *Aire*^{+/+} T cells. Therefore, it is difficult to interpret whether the reduction of T cells in *Aire*^{-/-} control host animals to a level similar to that observed in 3A9/InsHEL *Aire*^{-/-} mice is due to rescued negative selection or impaired positive selection. Further studies are required using other models to definitively prove that Aire regulates genes in the MECs that lead to thymic deletion of tissue-specific self-reactive T cells.

The role of TNF/TNFR and co-stimulatory family members in negative selection

Many studies have examined whether TNF/TNFR family members are involved in the negative selection of thymocytes. To date, this remains a controversial area [33–42]; see also Update). Although members of the TNF/TNFR family play a role in apoptosis, there is no convincing evidence that different family members participate in negative selection.

Recently, the role of TNF-related apoptosis-inducing ligand (TRAIL) in negative selection has been examined [43•]. TRAIL can interact with two receptors that contain intracellular death domains, known as death receptor 4

(DR4; TRAIL-R1) and DR5 (TRAIL-R2). TRAIL^{-/-} thymocytes were resistant to death induced by anti-CD3 in fetal thymic organ cultures. This anti-CD3-induced death could also be inhibited by soluble DR5 molecules. In addition, TRAIL^{-/-} thymocytes were refractive to endogenous superantigen (Mtv9)-mediated death. If there is defective negative selection in these animals, it may be predicted that they would display an increased incidence of spontaneous autoimmune disease, but this was not observed in these mice. TRAIL^{-/-} mice, however, were more susceptible to experimentally induced arthritis or diabetes. DR4 and DR5 signal through the Fas-associated death domain (FADD) and caspase-8 to induce apoptosis (Figure 1). However, another concern is that there is no evidence that FADD plays a role in thymocyte negative selection [44,45]. In addition, experiments using caspase-8-deficient thymocytes show that there is no protection from anti-CD3 mediated death *in vitro* [46]. The role of caspases and other related molecules in the pathway of thymocyte negative selection also remains controversial [47–54]. Therefore, despite evidence suggesting that TRAIL plays a role in thymocyte death, the known downstream pathways seem, at best, to have a controversial role in negative selection. It is

possible that TRAIL is able to induce death via another, as yet undefined, pathway.

There also remains a longstanding debate whether co-stimulatory molecules influence negative selection ([55] and references therein). New evidence suggests that B7-1 and B7-2 play a role in negative selection [55].

Apoptosis versus survival: playing with negative selection

The Bcl-2 family

Apoptosis is regulated by numerous pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. Recent studies have provided evidence that Bim, a Bcl-2 homology domain 3 (BH3)-only containing protein, plays a role in negative selection [56[•]]. Multiple models of thymocyte negative selection were examined, including anti-CD3-induced death, superantigen and antigen-specific deletion using CD4⁺/TCR transgenic models (OT-II) and CD8⁺/TCR transgenic models (H-Y). The authors concluded that Bim is required for thymocyte death, and that the lack of negative selection is the cause of autoimmune disease reported in these mice [57]. However, the authors have also acknowledged the importance of Bim in cytokine-mediated death [58]. It is not clear, therefore, whether negative selection of self-reactive T cells is prevented or delayed, or whether only the survival of DP cells is enhanced, possibly through a cytokine-dependent process.

Studies have suggested that Bim-mediated apoptosis requires both Bax and Bak [59,60]. Bax and Bak are pro-apoptotic members of the Bcl-2 family that have BH1, BH2 and BH3 domains. T cells deficient in both Bak and Bax (Bak/Bax-deficient cells) show an inhibition of the DN to DP transition [61[•]]. This is in contrast to the transgenic overexpression of Bax α , which leads to impaired positive selection, as shown by a limited number of cells differentiating from the DP to SP thymocyte stage [62]. The Bak/Bax-deficient mice develop an altered distribution of the proportion of thymocytes in each subset over time. In addition, thymocytes were resistant to multiple forms of death induced by gamma irradiation, etoposide, dexamethasone, anti-CD3 and superantigens. Fas-mediated death, however, was normal in the absence of Bak and Bax. [61[•],63]. Because Bak and Bax play a role in various death pathways in T cells, and because Bak/Bax^{-/-} thymocytes are resistant to many forms of apoptotic stimuli, it is difficult to interpret whether Bak and Bax play specific roles in negative selection. Bak/Bax^{-/-} mice develop severe splenomegaly and lymphadenopathy, primarily due to the accumulation of 'memory-type' lymphocytes [61[•],63]. Altered homeostasis is likely to be due to Fas-independent processes, as the induction of Fas death in thymocytes was similar to induction in controls. In addition, no spontaneous signs of autoimmunity, such as autoantibody production, were reported. The data

suggest that the absence of Bak/Bax leads to the impaired induction of lymphocyte apoptosis and, as a consequence, lymphocytes accumulate in secondary lymphoid organs.

It is noteworthy that the partial rescue of negative selection seen in the Bim- and Bak/Bax-deficient mice is similar to the phenotype observed in animals with defects in the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) signaling pathway. Mice lacking PTEN, a negative regulator of PI3K activity, or overexpressing PKB, an effector of PI3K that promotes survival, develop a polyclonal lymphoproliferative disorder accompanied by B-cell-mediated autoimmunity and inflammation (Figure 2; [64,65]). Further evidence suggests that this pathway, which includes Bim and Bax, is important in IL-7-mediated survival. Khaled *et al.* [66] have generated IL-7R α ^{-/-} Bax^{-/-} mice, and restored normal thymocyte development in four-week-old mice. Other studies have also linked IL-7R signaling with PI3K and PKB/Akt [67]. Collectively, these studies support the interpretation that the importance of this pathway is regulating survival at the DP stage, and not exclusively the mechanism of negative selection *per se*.

NF- κ B

The nuclear factor κ B (NF- κ B) family of transcription factors is known to promote survival and apoptosis in many different models. NF- κ B activity is regulated by a family of kinases called inhibitor of κ B (I κ B). Members of this family, such as I κ B α and I κ B β , form complexes with NF- κ B and retain NF- κ B in the cytoplasm. Upon the induction of various stimuli, such as stress, I κ B proteins become phosphorylated and are degraded via the ubiquitin pathway, releasing NF- κ B to the nucleus [68]. Interesting studies have recently identified a novel member of the I κ B family, called I κ B_{NS} [69^{••}]. Using representational difference analysis, Fiorini *et al.* [69^{••}] showed that this gene was upregulated upon TCR-induced negative selection. I κ B_{NS} binds to NF- κ B subunits and inhibits NF- κ B activity in *in vitro* assays. The overexpression of I κ B_{NS} in fetal thymic organ cultures reduced the percentage of DP and SP thymocytes and also accelerated anti-CD3-induced apoptosis. This suggests that I κ B_{NS} plays a key role in regulating thymocyte development and, potentially, negative selection.

Previous studies have shown that the NF- κ B transcription factor Rel does not play a role in thymocyte positive or negative selection using the H-Y TCR transgenic model bred onto the *c-rel*-deficient background [70]. Recent studies have examined the role of another family member, RelB, in thymocyte development. *RelB*-deficient mice have a two-to-threelfold reduction in thymic cellularity [71,72]. Guerin *et al.* [72] have shown that RelB is expressed in several subsets of thymocytes, including the DN, DP, immature CD4⁺ SP and immature CD8⁺ SP cells (but not the mature CD4⁺ or CD8⁺ SP thymocytes).

They propose that RelB is important for regulating the survival of these immature T cells. Previous studies have also shown that *RelB*-deficient mice develop rapid multiorgan inflammation and autoimmunity, and have a disrupted thymic medulla [71,73]. MECs are absent in the thymus of *RelB*^{-/-} mice, as are a subset of bone-marrow-derived dendritic cells (DCs; [73,74]). Earlier work has also shown that T cells specific for a viral superantigen are not deleted in the absence of RelB [8]. Therefore, there are multiple defects in these mice, and it is not yet clear whether autoimmunity is promoted by the absence of a subset of DCs or MECs, altered thymic architecture, defects in negative selection, or a combination of the above.

Signaling pathways and other transcriptional regulators involved in thymocyte apoptosis

Previous experiments have shown that JNK and p38 promote thymocyte death [75–77]. Interestingly, Bak and Bax have been linked to signals downstream of JNK/SAPK signaling, using Chinese hamster ovary (CHO) cells and mouse fibroblasts derived from gene-deficient lines (Figure 2; [78]). There is a possibility, therefore, that the mitochondrial apoptotic pathway is linked to JNK/SAPK signaling.

Recent experiments have clearly demonstrated that mitogen-activated protein (MAP) kinase kinase (MKK)6 plays an important role in p38-induced death (Figure 2). Using MKK6-deficient mice treated with anti-CD3, Tanaka *et al.* [79••] have shown that MKK6^{-/-} thymocytes are resistant to anti-CD3-induced death *in vivo*. This supports the importance of the p38 pathway in thymocyte death.

One of the most notable studies to investigate the mechanism of negative selection showed that the absence of Id3 (for inhibitor of differentiation/DNA binding) can strongly inhibit negative selection [11]. A family of Id proteins block the activity of a family of transcription factors called E proteins by forming heterodimers. Id3 can bind to E2A and prevent the transcription of E2A-regulated genes. Studies have shown that TCR signaling leads to an increase in Id3 expression [80]. This results in the formation of Id3–E2A heterodimers, which allows either positive or negative selection to occur. In the absence of Id3, negative selection of H-Y males was impaired, and a striking restoration of thymic cellularity and expression of the H-Y TCR was seen. However, the absence of Id3 also leads to impaired positive selection at the late SP stage. As a consequence, the H-Y TCR transgenic thymocytes ‘rescued’ from negative selection become arrested at the SP stage.

Although this study shows that Id3 can strongly inhibit negative selection, it also raises a conceptual question about the current models of negative selection, which suggest that ERK is involved only in positive selection

(Figure 2). Bain *et al.* [80] have shown that the Id3 transcripts are regulated downstream of the TCR by the ERK pathway. Although ERK is clearly involved in positive selection (reviewed in [2]), studies have also shown that ERK is activated by negative selecting stimuli [81,82] and modulates the thresholds for positive and negative selection [83,84]. Further studies are needed to gain insight into these observations.

Previous work has shown that the orphan nuclear receptor Nur77 is involved in thymocyte death [85–88]. Recent studies have shown that a histone deacetylase HDAC7 is important for regulating Nur77 transcription in DP thymocytes [89•]. This identifies new molecules and provides new insights into the regulation of thymocyte death.

Concluding remarks

Although interpretations in the literature suggest that impaired negative selection is linked to the development of autoimmunity, many animal models that have been studied may, in fact, represent multiple ways of developing autoimmunity. The clearest direct association between impaired negative selection and human autoimmunity is in the studies of the Aire^{-/-} mice [30•,31••]. Other animal models that are susceptible to autoimmunity have also been associated with impaired negative selection ([90–92]; see also Update). At present, there is limited evidence to suggest that other gene-deficient mice that develop autoimmunity do so by truly disrupting negative selection, rather than by altering the survival of thymocytes, lymphocytes or other mechanisms. Promoting survival and perturbing homeostasis have previously been linked with autoimmunity [93,94]. As the mechanisms of negative selection are elucidated, the models that relate negative selection and autoimmunity will be clarified.

Update

Recent work has further examined the importance of IL-7 during thymocyte development [96]. In addition, further studies have been published which adds to the controversial data for the mechanisms underlying negative selection [97,98].

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
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