Good riddance: thymocyte clonal deletion prevents autoimmunity

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Clonal deletion is arguably the most important mechanism of eliminating self-reactive thymocytes from the T-cell repertoire. Recent work has identified new players in this process. On the thymocyte side, several molecules have been newly implicated in the pathway from initial T-cell receptor signaling through to the final result: gene transcription and thymocyte apoptosis. In addition, several proapoptotic molecules have been found to be necessary for the death of self-reactive thymocytes. On the antigen-presenting cell side, the expression of peripheral self-antigens, regulated at least in part by the autoimmune regulator (AIRE) protein, is crucial for complete elimination of autoreactive thymocytes. The importance of thymic peripheral antigen expression and clonal deletion to self-tolerance is demonstrated in the autoimmune diseases autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy and type-1 diabetes mellitus.

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Abbreviations

AIRE	autoimmune regulator
APC	antigen-presenting cell
APECED	autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy
DP	$CD4^+CD8^+$ double positive
FTOC	fetal thymic organ culture
HDAC	histone deacetylase
HEL	hen-egg lysozyme
IκB	inhibitor of κB
LTβR	lymphotoxin β receptor
MEC	medullary epithelial cell
MEF2D	myocyte enhancer factor 2D
NOD	non-obese diabetic
OVA	ovalbumin
RARα	retinoic acid receptor α
TCR	T-cell receptor

Introduction

As thymocytes differentiate into mature αβ T cells they are subject to several selection events. These thymic processes ensure that mature T cells are capable of recognizing foreign peptides in the context of self-MHC molecules, but not of reacting to self-peptide–MHC molecule complexes, thereby preventing autoimmunity. So-called 'β-selection' results from survival signals transmitted through the pre-T-cell receptor (pre-TCR), including a competent TCR β chain. Positive selection results from αβ TCR recognition of self-peptide–MHC molecule complexes generally presented by thymic cortical epithelial cells; in the absence of such recognition, thymocytes die by neglect. However, if recognition of the complexes, whether displayed on cortical or medullary epithelial cells, dendritic cells or macrophages, is too strong, then those self-reactive thymocytes are negatively selected. Clonal deletion is one of the mechanisms — probably the most significant— of negative selection, culminating in death by apoptosis.

The process of clonal deletion is initiated by interactions between cell-surface molecules on a thymic stromal antigen-presenting cell (APC) and a differentiating thymocyte. Distinct signaling pathways are engaged in the thymocyte, promoting transcription, translation and/or post-translational activation of molecules that mediate apoptosis. The identities of many of the molecules involved in the clonal deletion process are still unknown; however, work over the past two years has pointed to some intriguing candidates on both the thymocyte and APC sides (Figure 1), and we have begun to elucidate their precise roles. We will discuss some of these recently identified molecules, highlighting in particular the implication of certain molecules in autoimmune diseases such as autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also called autoimmune polyglandular syndrome-type 1 [APS-1]) and type-1 diabetes mellitus.

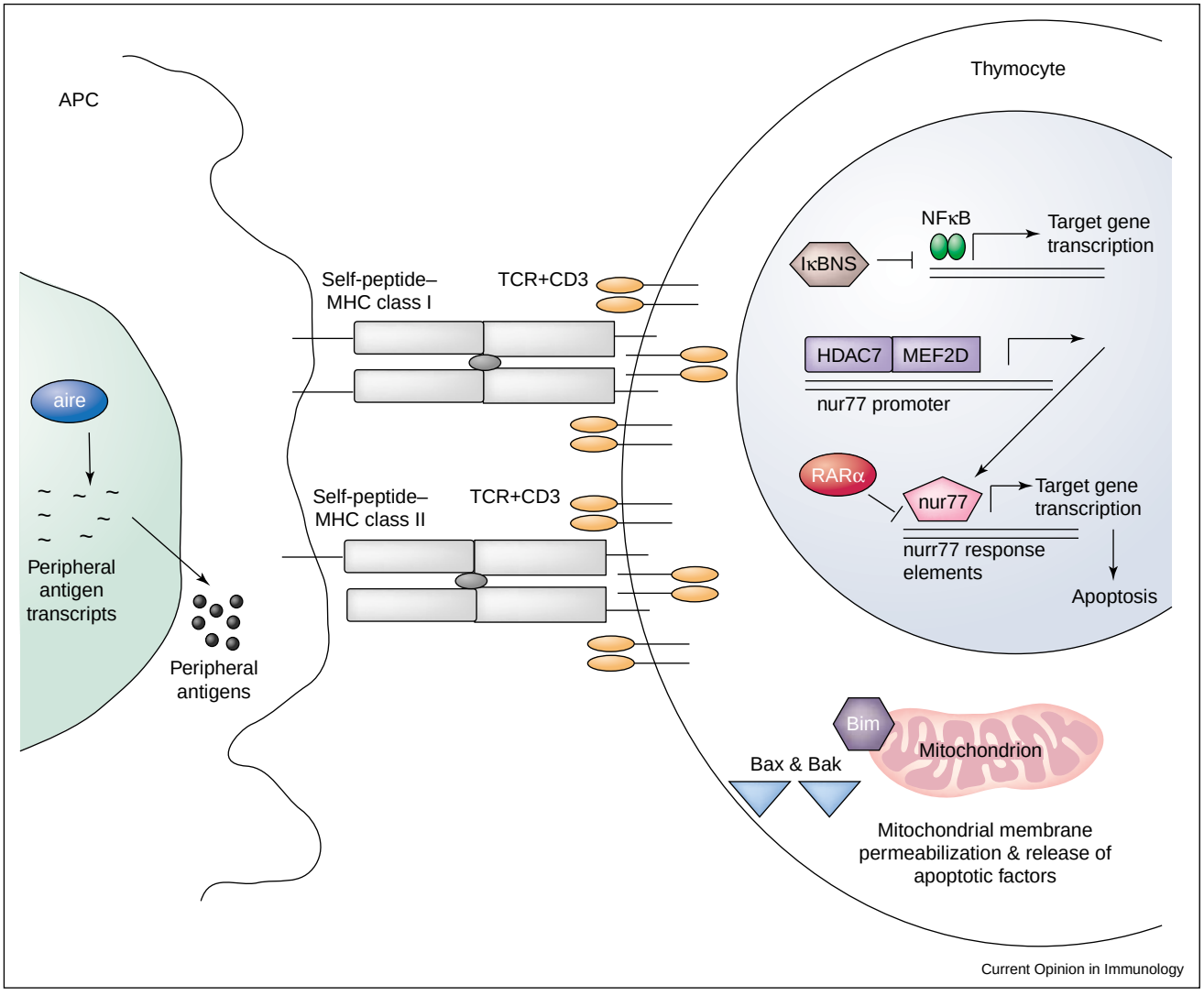
The thymocyte: intracellular signaling molecules and transcription factors

Three important new leads concerning the signaling pathways engaged in thymocytes undergoing clonal deletion have been provided in the past two years.

IκBNS

By performing representational difference analysis on thymocytes from TCR-transgenic mice treated or not treated with agonist peptide, Fiorini and colleagues [1••] identified a new inhibitor of κB (IκB) molecule, IκBNS, selectively expressed by thymocytes undergoing TCR-mediated clonal deletion and especially upregulated following TCR engagement. Their interest was piqued by IκBNS because it is quite similar to other IκB family members, it binds NF-κB *in vitro* and inhibits its transcriptional activity. This is highly relevant given the

Figure 1



Thymocyte clonal deletion. Thymic antigen-presenting cells (APCs; cortical and medullary epithelial cells, dendritic cells and macrophages) present self-peptides to developing thymocytes. In the nucleus of the medullary epithelial cell (MEC; light green) the aire protein promotes the expression of peripheral self-antigens. A high-affinity interaction of MHC class I or class II on the APC with the T-cell receptor–CD3 complex on the thymocyte initiates clonal deletion. Downstream, in the nucleus (light blue), IκBNS inhibits NFκB activity, presumably facilitating thymocyte apoptosis. The HDAC7–MEF2D complex promotes nur77 expression. The nur77 protein binds nur77 response elements, leading to the transcription of genes that cause apoptosis. This binding is inhibited by RARα. Bim, Bax and Bak act in concert to disrupt the mitochondrial membrane, leading to a release of pro-apoptotic factors and eventual apoptosis.

well-known role of NF-κB in promoting lymphocyte survival [2]. In fetal thymic organ cultures (FTOCs) seeded with T-cell progenitors ($CD4^-CD8^-$ double-negative) that were transduced with a retrovirus driving IκBNS expression, thymocyte differentiation was altered. There were fewer $CD4^+CD8^+$ double positive (DP) and $CD4^-$ and $CD8^-$ single positive thymocytes among the IκBNS-transduced cells, suggesting an increase in their clonal deletion. IκBNS-transduced cells were also more likely to die following addition of anti-CD3ε antibody to the FTOCs, confirming an increased sensitivity to TCR-mediated deletion.

Histone deacetylase 7

Histone deacetylase 7 (HDAC7) is an another nuclear factor involved in clonal deletion [3••]. HDACs play a key role in the regulation of gene expression by modulating the acetylation state of nucleosomal histones. Deacetylation of histones causes the associated DNA to become more tightly wrapped around the nucleosome, leading to decreased transcription factor accessibility and thereby to decreased gene transcription [4]. HDAC7 is expressed most highly in DP thymocytes of more mature phenotype ($CD3^{med/hi}$). It interacts with myocyte enhancer factor 2D (MEF2D), a transcriptional regulator previously shown to

influence expression of nur77. Several studies have implicated the orphan steroid receptor nur77 (and/or the related family members nurr1 and nor1) in thymocyte clonal deletion [5]. *In vitro* assays demonstrated that the HDAC7–MEF2D interaction at the *nur77* promoter repressed nur77 expression and led to decreased apoptosis in response to TCR triggering via anti-CD3 antibody treatment.

Retinoic acid receptor α

Another newly identified signaling molecule with potential involvement in clonal deletion is retinoic acid receptor α (RARα), a nuclear retinoid receptor expressed in the thymus. It was previously shown that the treatment of thymocytes with retinoic acid inhibits TCR-mediated thymocyte apoptosis *in vitro* [6]. Mice treated with the synthetic RARα ligand CD336 exhibited decreased thymocyte apoptosis following treatment with either anti-CD3 antibody or agonist peptide (in the case of TCR transgenic mice; [7•]). CD336 was also shown to have an effect on the function of nur77. Simultaneous treatment of mice with agonist peptide and CD336 blocked binding of nur77 to its known DNA target sequences. Thus, it appears that RARα may operate to keep thymocyte apoptosis in check through inhibition of nur77. However, it remains to be elucidated how specific the CD336 inhibitor is for RARα, and whether the effect on nur77 and apoptosis might not emanate through another molecule.

The thymocyte: proapoptotic molecules

Most of the recent data on the apoptotic pathways involved in thymocyte clonal deletion argue for the dominance of the intrinsic pathway, which results in caspase-9 activation, rather than the extrinsic pathway, which culminates in caspase-8 activation. It has been shown that a dominant-negative version of the Fas-associated death domain protein, an adaptor situated downstream of most death-signaling cell-surface receptors of the extrinsic pathway, did not have a significant effect on thymocyte clonal deletion [8]. Recently, the TNF-related apoptosis-inducing ligand, a member of the TNF family and the extrinsic pathway, was reported to promote the deletion of self-reactive thymocytes [9], but this has been disputed by another group [10]. On the other hand, several members of the intrinsic pathway have been shown to play a crucial role in thymocyte clonal deletion. Bim, Bax and Bak are all Bcl-2 family members that have a proapoptotic function in multiple cell lineages. Bim-deficient and $Bax^{-/-}Bak^{-/-}$ double-deficient thymocytes both exhibited clonal deletion abnormalities in several experimental systems [11••,12••]. Thymocytes with either defect (Bim deficiency or Bax/Bak double-deficiency) were resistant to deletion mediated by anti-CD3 antibody or endogenous superantigens. In addition, in the OT-1 and HY TCR transgenic systems, *bim* knockout TCR transgenic thymocytes were resistant to cognate antigen-induced clonal deletion. It was previously shown that Bax and Bak are required for the proapoptotic function of Bim [13,14]. Because of the similar impaired clonal deletion phenotypes in the *bim*-knockout and $bax^{-/-}bak^{-/-}$ double knockout thymocytes, it is likely that these factors also work together to promote apoptosis of thymocytes. Interestingly, Bim is also required for apoptosis of activated T cells [15] and autoreactive B cells [16].

The thymocyte: gene expression profiling of clonal deletion

Two groups have recently performed global gene expression profiling studies of thymocytes undergoing clonal deletion in TCR transgenic systems [17•,18•]. Both groups used Affymetrix GeneChips® to examine the expression profiles of DP thymocytes from TCR transgenic mice that had been injected with cognate peptide 0.5–8 hours before. These profiles were compared with those of control DP TCR transgenic cells from mice that had not been injected with cognate peptide [18•], or from mice bearing a nonselecting MHC [17•]. The groups found that a number of genes were differentially expressed in the cells undergoing deletion, including several genes already known to be involved in clonal deletion, such as nur77 and Bim, as well as genes involved in apoptosis, transcription and cell–cell interactions. There are many interesting candidate genes on the lists from both groups, and follow-up experiments to determine which of these genes are truly important players in clonal deletion should be performed.

The antigen-presenting cell: peripheral antigen expression and aire

The big news on the APC side of things has been the role of thymic medullary epithelial cells (MECs) in central tolerance. It has become clear over the past few years that the thymic stroma expresses several peripheral tissue-specific antigens, and that the expression of these antigens is important for purging the repertoire of self-reactivity [19]. Many of these antigens are expressed by thymic epithelial cells, in a small subset of MECs in particular. MECs are also the major expressors of the autoimmune regulator (AIRE) protein, a putative transcriptional control element [20]. Mutation of the *AIRE* gene in human patients is the cause of the multiorgan autoimmune endocrine disease APECED [21,22]. Because AIRE and peripheral antigens were both expressed in MECs, it was speculated that AIRE might control the transcription of these antigens. To generate a mouse model for APECED and to further study AIRE's function, investigators have independently generated two lines of *aire* knockout mice [23••,24••]. Both lines exhibited lymphocytic infiltrates and autoantibodies directed against specific structures of many peripheral organs and tissues, including the salivary gland, retina, pancreas, stomach, ovary and thyroid. It was found, through bone marrow chimera and thymic transplant experiments, that the non-hematopoietic cells of the thymic stroma are the

only cells that must bear the *aire* mutation in order for disease to occur [24••]. Gene expression profiling of sorted MECs from the *aire* knockout mice and wild-type littermates revealed that, in the knockouts, expression of many peripheral antigen genes was depressed. These genes encompassed many expressed in the targeted organs of *aire* knockout mice, and also included two antigens, preproinsulin and cytochrome p450 1a2, against which some APECED patients produce autoantibodies.

The thymi of *aire* knockout mice appear normal, in terms of thymocyte numbers and subset percentages, but, because *aire* knockout mice and APECED patients both exhibit autoimmune disease, there had to be impairment in tolerization of autoreactive thymocytes, which was likely to be related to the defective expression of peripheral antigens by the thymic stroma. Negative selection of thymocytes in *aire* knockout mice has more recently been studied in TCR transgenic systems. The 3A9 TCR recognizes a peptide from hen-egg lysozyme (HEL) bound to I-E^k. In mice that express the 3A9 TCR and HEL under the control of the rat insulin promoter, which drives HEL expression in both the pancreas and in MECs, the transgenic thymocytes were clonally deleted. Breeding aire knockout mice with HEL-3A9 transgenic mice yielded offspring demonstrating decreased clonal deletion of 3A9 thymocytes and increased numbers of these cells in the thymus and the periphery [25•]. This suggests that the regulation of peripheral antigen expression in MECs by aire controls thymocyte deletion; however, it was not demonstrated that aire actually regulates thymic HEL expression. Similar experiments have been performed using transgenic mice expressing either the OT-1 or OT-2 transgenic TCR plus the antigen chicken ovalbumin (OVA) under the control of the rat insulin promoter (MA Anderson *et al.* unpublished). Breeding aire knockout mice with OVA–OT-1 or OVA–OT-2 transgenic mice led to offspring that exhibited impaired clonal deletion of the transgenic cells. Strikingly, in the OVA–OT-1 system, *aire* knockout mice were born with diabetes, thereby making a direct link, in at least one system, between aire expression, impaired central tolerance and autoimmunity.

The central tolerance defect observed in *aire* knockout mice could have resulted from impaired clonal deletion, defective positive selection of regulatory T cells, or a combination of the two. The two studies cited above demonstrate that clonal deletion is indeed defective in *aire* knockout mice but does not rule out an additional deficiency in regulatory T cells. In the HEL–3A9 and OVA–OT-1 and -2 systems, there was no significant difference in the number of $CD4^+CD25^+$ regulatory T cells in the peripheral repertoire in the presence or absence of aire [25•]. $CD4^+CD25^+$ cells from *aire* knockout mice also exhibited normal suppressive function *in vitro* and *in vivo*, the latter most evident from dual thymus (*aire* wild-type plus *aire* knockout) transfer experiments (MA Anderson *et al.*, unpublished).

Recently, it was proposed by Chin and colleagues [26] that lymphotoxin drives the expression of aire in the thymus. In lymphotoxin β receptor (LTβR) and in lymphotoxin α knockout thymi, the level of aire mRNA is lower than in wild-type thymi. This may be due to an effect of lymphotoxin on *aire* transcription, but it may also be due to a defect in development of the knockout thymi, causing fewer aire-expressing cells to develop in the first place. Boehm and colleagues [27] counted the UEA-1^+ (aire-expressing) MECs in the thymi of LTβR knockout mice and found a significant decrease in the number of cells in the knockout versus in wild-type littermates, a finding in disagreement with the nonquantitative UEA-1 immunofluorescence data in [26]. Chin and colleagues [26] also observed that, when mice were injected with an agonistic anti-LTβR antibody, aire mRNA expression, as well as expression of the peripheral antigen insulin 1, was induced within 8 hours. This interesting finding should be investigated further, to determine the mechanism of the aire and insulin 1 mRNA inductions in this system.

Thymic insulin expression and clonal deletion in the non-obese diabetic mouse

The importance of peripheral antigen expression in the thymus to actual tolerance induction has recently been examined in another autoimmune disease model, spontaneous type-1 diabetes in the non-obese diabetic (NOD) mouse. It was previously shown that the level of insulin expression in the human thymus correlated with the number of tandem repeats of a 14 base-pair unit in the promoter region of the *insulin* gene (IDDM2), which, in turn, correlated with diabetes susceptibility. A higher thymic insulin level associated with protection against diabetes, whereas a lower level associated with diabetes susceptibility [28,29]. In contrast to humans, who only have one *insulin* gene, mice express two forms of insulin encoded by separate genes. Both the *insulin 1* and *2* genes are expressed in pancreatic islet β cells, but *insulin 2* is expressed at a much higher level than *insulin 1* in the thymus. To examine what effect thymic insulin level might have on the selection or deletion of insulin-reactive T cells, Chentoufi and Polychronakos [30••] produced mice carrying different numbers of insulin alleles. They observed a decrease in the thymic insulin levels corresponding to the decrease in number of insulin alleles: $ins1^{+/+}2^{+/+}$ > $ins1^{-/-}2^{+/+}$ > $ins1^{-/-}2^{+/-}$ > $ins1^{+/+}2^{-/-}$ > $ins1^{+/-}2^{-/-}$. Pancreatic insulin levels, however, remained constant. Splenic T cells from $ins1^{+/+}2^{-/-}$ mice were more reactive to insulin than $ins1^{-/-}2^{+/+}$ cells, presumably because the $ins1^{+/+}2^{-/-}$ cells were exposed to less insulin in the thymus.

Proinsulin 2 knockout mice have been made [31] and backcrossed to the NOD genetic background. On the

NOD background, the knockout mice exhibited earlier, more severe insulitis and significantly earlier diabetes than wild-type NOD mice. T cells from the knockout mice were reactive to the proinsulin 2 peptide 88–103, an epitope shared with proinsulin 1, to which the wild-type mice were not reactive [32••]. This lends additional support to the idea that when an antigen is not expressed at a high enough level in the thymus, some thymocytes specific for that antigen may escape clonal deletion. These cells can cause autoimmunity when they mature and migrate to the periphery.

Interestingly, *proinsulin 1* knockout mice on the NOD background had the opposite phenotype: almost all knockout mice were free of insulitis and diabetes [33]. Perhaps this is because insulin 1 is the primary target of insulin reactivity in the NOD periphery, although it is not yet clear why it might be preferred over insulin 2 or other antigens. NOD mice may have other, non-insulin-specific clonal deletion defects as well.

Two recent studies have demonstrated impaired clonal deletion in NOD mice. In one study, semi-mature (heat stable antigen high, $CD4^+CD8^-$) NOD thymocytes were less susceptible to apoptosis than thymocytes from several other autoimmune-prone strains, when treated with anti-TCRβ and anti-CD28 monoclonal antibodies *in vitro*. Similar results were obtained *in vivo*, when NOD and B6 mice were injected either with anti-TCRβ and anti-CD28 antibodies or with superantigen. More semi-mature thymocytes survived these treatments in the NOD thymus than in the B6 thymus [34]. These data have been disputed by another group, however, who performed similar experiments by treating NOD and B6 semi-mature thymocytes with anti-CD3 and anti-CD28 antibodies *in vitro* and *in vivo*. They observed little difference in the level of apoptosis between the two strains [35]. It is not yet clear what caused this discrepancy, although it may be due to the different methods of purifying the thymocytes or to the different antibodies used by the two groups. In the second study, the HEL–3A9 TCR transgenic system described above was used to study clonal deletion in NOD mice bearing the selecting MHC H-2^k. In HEL–3A9 NOD–H-2^k mice, significantly less clonal deletion occurred than in 3A9/HEL B10–H-2^k mice. As a result, there were more 3A9 clonotype-positive mature T cells in the periphery of the NOD transgenic mice. By performing bone marrow chimera experiments, the authors determined that the impaired clonal deletion was intrinsic to the NOD thymocytes [36].

Conclusions

Clearly, impaired clonal deletion can result in the survival of self-reactive T cells and, often, in their response to the self-constituent in the periphery. Thus, central tolerance via thymocyte clonal deletion is a crucial mechanism for preventing pathogenic immune responses to self. However, it is not the only important mechanism, as demonstrated by the equally severe autoimmune phenotype of mice and humans with a mutated *Foxp3* gene, which is essential for the correct activity of $CD4^+CD25^+$ regulatory T cells.

Although several important players in the clonal deletion process have recently been identified, much work remains to be done before a coherent scenario can be constructed. On the thymocyte side, many components of the pathway from TCR triggering through the apoptosis of autoreactive thymocytes are unidentified, and links between the different components have yet to be drawn. On the stromal side, the exact roles of each thymic stromal cell type in self-antigen presentation and clonal deletion (as well as other forms of negative selection) are still unclear.

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