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News and Commentary

Suicide determines self

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Apoptosis plays a prominent role in the maintenance of self-tolerance in the immune system. A young adult mouse with 100–200 million thymocytes generates between 20–40 million new T cells per day. But the number of T cells that leave the thymus and enter the peripheral T cell pool is only about 2–3% of the number initially generated[1] indicating that massive death must occur. Thymocytes are removed through apoptosis during their development. However, the precise molecular mechanisms of this deletion are just being elucidated.

Immature thymocytes are subjected to stringent selection. Thymocytes that fail to receive a signal via their T cell receptor (TCR) will undergo apoptosis (death by neglect). Only those thymocytes expressing a T cell receptor capable of interacting with non-self-peptide presented by MHC molecules on thymic epithelial cells are positively selected and migrate to the periphery. In contrast, thymocytes with receptors that bind to self-peptide-MHC complexes with high affinity (autoreactive thymocytes) are negatively selected and die. Hence, negative selection ensures tolerance to normal tissue and prevents autoimmunity. Negative selection involves thymocytes at the immature $CD4^+CD8^+$ double positive (DP) development stage or early in the mature single positive stage $CD4^-CD8^+$ or $CD4^+CD8^-$ (SP). Although negative selection of thymocytes is clearly mediated by apoptosis,[2,3] researchers have been searching for a decade to what may be called the 'Holy Grail of thymocyte biology', i.e. the key regulators of this process. This may now have come to an end as Bouillet *et al*.[4] in a recent report, have identified a new player in the killing fields of negative selection.

Apoptosis occurs either as a consequence of extracellular signalling events, such as crosslinking of surface receptors, or via the mitochondrial pathway (Figure 1). Surface receptors that induce apoptosis belong mostly to the Tumour Necrosis Factor receptor (TNF-R) superfamily and there is ample evidence to suggest that the TNF-R family is involved in peripheral tolerance, that is tolerance induced outside the thymus. Indeed, CD95 (APO-1/Fas) has been shown to mediate peripheral tolerance as a natural mutation disrupting its death-signalling function causes lymphadenopathy and autoimmune disorder. As central and peripheral tolerance are both induced via TCR triggering, these observations hinted to a role for the TNF-R family in central tolerance. However, its involvement in central tolerance has so far remained controversial. Initially most investigators agreed that the CD95 system is not involved in negative selection because the TCR repertoire in mice with a defect in this system (lpr and gld) was not altered. But on closer inspection it was found that negative selection might involve CD95 when T cells encounter high antigen concentrations at the semi-mature SP stage of development.[6] Controversial results have also been obtained for other death receptors in their role during negative selection such as TNF-R1[7] and CD30.[8,9] Furthermore, it was demonstrated that receptors for TRAIL are not major players.[10] Finally and very convincingly, dominant negative FADD, the common signalling molecule downstream of TNF-R did not affect thymic deletion, which ruled out a major role for the death receptors in deletion.[11,12]

The alternative pathway of death is the mitochondrial pathway, which is controlled by members of the Bcl-2 family. There are anti-apoptotic members of this family: Bcl-2 and Bcl-X_L among others, and pro-apoptotic members, which include molecules such as Bax and Bak. Low levels of anti-apoptotic Bcl-2 have been detected in DP thymocytes, upregulation is observed upon TCR signalling; whereas the proapoptotic Bcl-X_L is expressed at high levels in DP. This pattern of expression suggests that the Bcl-2 family could be involved in the regulation of negative selection. However, results obtained with mice transgenic for Bcl-2 showed controversial results: superantigen-mediated thymocyte deletion was inhibited in some studies but not in others.[13] In addition, antigenic deletion was inhibited in TCR transgenic mice that overexpressed Bcl-2 in foetal thymus organ cultures but not in TCR transgenic mice.[15] Exogenous expression of Bcl-X_L had no effect on negative selection[16,17] and neither did the pro-apoptotic Bcl-2 family member Bax.[14] The new study by Bouillet *et al*.[4] now provides compelling evidence that mice deficient for Bim, a pro-apoptotic Bcl-2 family member, have impaired negative selection induced by superantigen, antigen and anti-CD3.

Bim belongs to the BH3-only fraction of the Bcl-2 family. This subfamily of small proteins serve as sensors for all sorts of cellular stress signals and have the capacity to unleash the mitochondrial apoptotic machinery (for a review see Huang *et al*.[18]), Bad, for instance, is phosphorylated in healthy cells and is thereby sequestered by the scaffold protein 14-3-3. Cytokine depriviation is 'sensed' by Bad after which it loses its phosphorylation. This sets Bad free to translocate to the mitochondria where it interferes in the careful balance between pro and anti-apoptotic Bcl-2 family

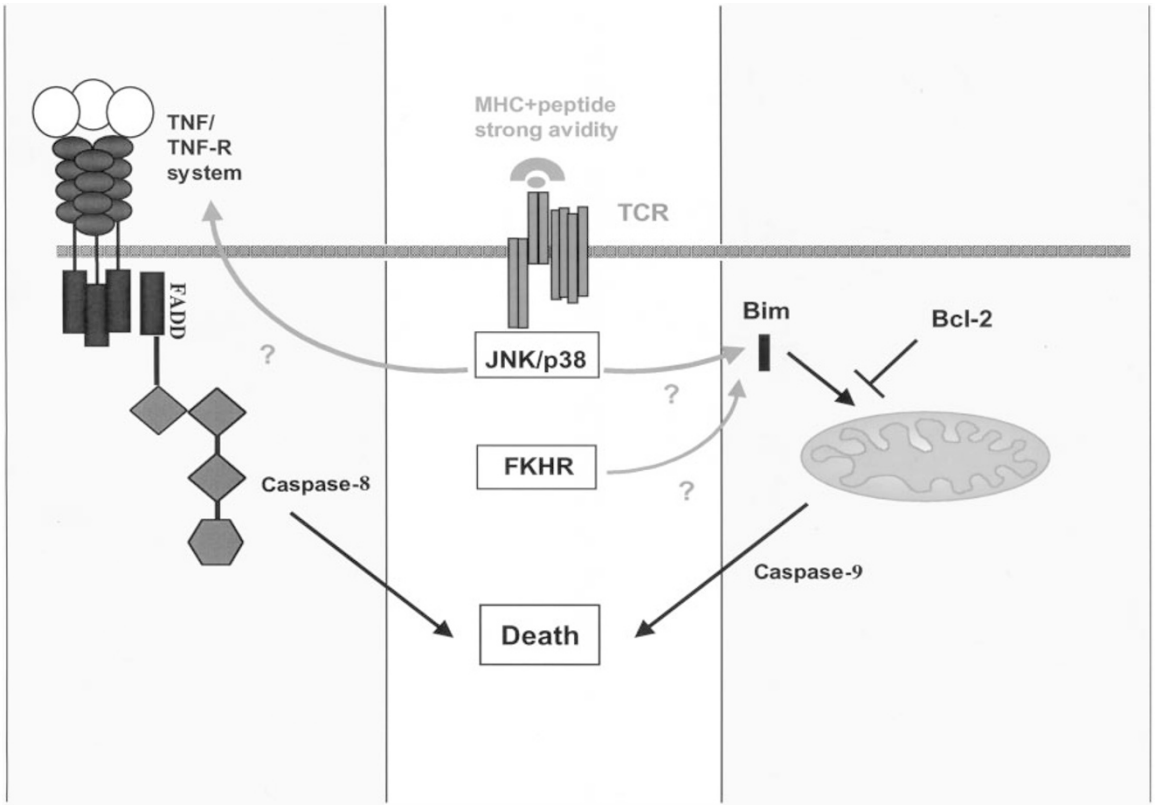

Figure 1 Possible signalling routes leading to negative selection. High affinity interactions at the thymocyte double positive stage trigger the activation of bim on one hand and the expression of TNF family members on the other. Combined, these effects signal apoptosis of the auoreactive thymocytes. The involvement of the protein kinase JNK and the transcription factor FKHR remain undefined but both are interesting candidates to serve as signalling intermediates

members. Such mitochondrial translocation is a common theme in the action of this family of small proteins. Bid, which normally resides in the cytoplasm as an inactive molecule translocates to the mitochondria upon cleavage by caspase-8 or granzyme B. Bim itself was reported to translocate to the mitochondria from the cytoskeleton upon cytokine deprivation. What then happens at the mitochondria? Several models have been put forward to explain the pro-apoptotic effects of BH3-only proteins (for a review see Strasser *et al*.[19]). One relies on the fact that the BH3-only proteins can target anti-apoptotic Bcl-2 family members like Bcl-2. Binding leads to 'inactivation' and then leaves the pro-apoptotic members, Bax and Bak, to perform their deadly mission. Alternatively, binding to the anti-apoptotic members could release APAF-1 sequestered by Bcl-2 and signal apoptosis by direct caspase-9 activation. Recent data, however, suggest that APAF-1 is not required for the BH3 molecules to exert their deadly act.[20] More importantly, in the absence of Bax and Bak no induction of apoptosis is detectable despite high levels of active BH3-only killers. This indicates that it is very unlikely that the BH3-only proteins directly activate the APAF-1 cascade but instead require mitochondrial disruption induced by Bax or Bak. A model to integrate all these findings was put forward by Cheng *et al*.[20] They suggest that anti-apoptotic Bcl-2 family members serve as a sink for the BH3-only proteins. Once this sink overflows the BH3-only proteins set off to aid the pro-apoptotic ones in their mitochondrial membrane disruption. This then leads to release of all apoptotic inducers from the mitochondria and results in the activation of caspase-dependent and independent death pathways. Although this is a compelling model, the question that remains is how very few BH3-only proteins (<1000 Bim molecules/lymphoid cell) can effectively trigger apoptosis in a cell that expresses a lot of anti-apoptotic molecules. To make this model stick, one has to assume that filling the sink is not a simple one-on-one event but should be 'infectious', i.e. filling up one sink changes several others in a way that inactivates them. Whether this is true remains to be seen, but it certainly does provide an explanation for one striking difference in negative selection observed in Bim knockout mice as compared to mice that overexpress Bcl-2 or Bcl-X_L. As mentioned, the latter anti-apoptotic molecules only marginally affect negative selection of thymocytes,[13,16] while Bim seems responsible for a large fraction of death observed in autoreactive T cells. If indeed, small numbers of Bim can inactivate large numbers of anti-apoptotic family members this is the expected outcome. Nevertheless, one cannot exclude that Bim has a, until yet, unidentified second function that promotes cell death. In addition, inactivation of Bcl-2 or Bcl-X_L by post-translational modification could occur during negative selection allowing for inactivation of a large fraction of these molecules in a Bim-independent fashion.

Whatever the reason, it is clear that Bim is a potent killer in thymocytes, yet its regulation remains an enigma. Compelling evidence is provided by Bouillet *et al*.[4] to indicate that TCR triggering of thymocytes increases the expression of Bim by about threefold. In addition, this coincides with a clear increase in the amount of Bcl-X_L/Bim complexes, suggestive for the induction of apoptosis. But how then is Bim expression induced? A large amount of knockout and transgene studies have indicated a role for the JNK/p38 pathway downstream of TCR triggering in negative selection. For instance, Grb2 haploid insufficiency in thymocytes weakens TCR-induced JNK and p38, but not MAPk, activation and ablates negative selection.[21] Similarly, expression of dominant negative JNK prevents negative selection,[22] while thymocyte expression of active Rac, a small Ras-like GTPase that can drive JNK and p38, shifts thymic selection from positive to negative.[23] This combined with the fact that JNK can promote expression of Bim in neurons,[24] suggests that it may be directly upstream of Bim in thymocytes as well. Unfortunately, recent experiments performed by the group of Flavell have marginated the role of JNK in thymocyte development[25] and it therefore seems unlikely to be the upstream regulator of Bim.

Similar to JNK, the forkhead transcription factor FKHR-L1 is capable of driving transcriptional activation of the *bim* gene and this occurs when cells are deprived of cytokines.[26] This event requires dephosphorylation of this transcription factor. Under favourable cytokine conditions phosphorylation and thus inactivity of FKHR-L1 is assured by the activity of protein kinase B (also called Akt), a kinase that is activated by PI-3kinase. An important argument that speaks for such a regulatory mechanism in thymic negative selection is the fact that thymocytes deficient for PTEN, a phosphatase that counterbalances PI-3kinase, display increased PKB activity and fail to delete autoreactive thymocytes.[27] Nevertheless, Jones *et al*.[28] clearly showed that although exogenous expression of active PKB could increase survival of thymocytes it was incapable of preventing negative selection. More importantly, both models rely on transcriptional activation of Bim, while Bouillet *et al*.[4] show that Bim mRNA levels are unaffected. Instead a post-transcriptional modification is proposed to explain the elevated levels of Bim, but unfortunately, at this point it remains a puzzle what such a modification would consist of or how it is induced. However, both questions will need to be answered in order to fully understand the effects of Bim on negative selection.

Does this mean the search for key regulators can be called off? We fear not; first of all it is essential to note that although the effects reported by Bouillet *et al*.[4] are impressive they are by far not 100%. Moreover, two other recent studies have given the TNF-R superfamily another chance for their involvement in thymic clonal deletion. Wang *et al*.[29] reported that deletion induced by antigen or anti-CD3, but not by superantigen, is impaired in mice deficient for DR3. Similarly, a role for LIGHT in negative selection was put forward.[30] Whether these effects are interconnected awaits further experiments, but they clearly indicate that there is more to negative selection than activation of Bim. Indeed, when Bim would be the sole player, Bim deficiency should result in the generation of autoreactive T cells that would trigger severe signs of autoimmunity. Bouillet *et al*.[4] have tried to analyze this in the well-known HY-model and found no clear evidence to support this notion. HY-specific TCR transgenic T cells are normally negatively selected in male (HY^+) mice, while females (HY^-) positively select these T cells. In the male Bim$-/-$ background, however, these HY-specific T cells largely escape deletion. Strangely enough these T cells can now be detected in the periphery at numbers that even exceed the ones detected in female mice. Nevertheless, it seems they are still not behaving as true autoimmune cells as the mice seem to survive this ordeal. Careful analysis of these T cells indicates that the lack of autoreactivity likely results from a reduced CD8 expression, which prevents their activation. More importantly, this is one of the classical signs of autoreactive T cells that have only partially escaped negative selection.

It is true though that *bim* knockout mice develop autoimmunity.[31] Nevertheless, this is largely due to autoreactive B and not T cells. One can obviously not exclude that this simply reflects a more rapid induction of B cell-mediated autoimmunity that overshadows the T cell arm, but it seems more reasonable to assume that T cells meet a second barrier that prevents them from becoming self destructive. Apparently, Bim deficiency is not sufficient to allow for complete T cell maturation of autoreactive T cells, indicating that an additional barrier must be taken before such T cells can arise. Whether 'wrongly' selected thymocytes meet their second barrier before they can leave the thymus or whether this is due to peripheral deletion awaits further analysis. It is clear though that Bim is one of the keyplayers that prevents our body from becoming a prey for autoreactive T cells, but more players are likely to follow.

1. Chen *et al*. (1983) Thymus 5: 179–185
2. Murphy *et al*. (1990) Science 250: 1720–1723
3. Surh *et al*. (1994) Nature 372: 100–103
4. Bouillet *et al*. (2002) Nature (in press)
5. Sebzda *et al*. (1999) Annu. Rev. Immunol. 17: 829–874
6. Kishimoto *et al*. (1998) J. Exp. Med. 187: 1427–1438
7. Page *et al*. (1998) J. Immunol. 160: 120–133
8. Amakawa *et al*. (1996) Cell. 84: 551–562
9. DeYoung *et al*. (2000) J. Immunol. 165: 6170–6173
10. Simon *et al*. (2001) Proc. Natl. Acad. Sci. USA 98: 5158–5163
11. Newton *et al*. (1998) EMBO J. 17: 706–718
12. Walsh *et al*. (1998) Immunity 8: 439–449
13. Williams *et al*. (2001) Trends Immunol. 22, 107–111
14. Williams *et al*. (1998) J. Exp. Med. 188: 1125–1133
15. Strasser *et al*. (2000) Annu. Rev. Biochem. 69: 217–245
16. Grillot *et al*. (1995) J. Exp. Med. 182: 1973–1983
17. Chao *et al*. (1997) Int. Immunol. 9: 1375–1384
18. Huang *et al*. (2000) Cell 103: 839–842
19. Strasser *et al*. (2000) Annu. Rev. Biochem. 69: 217–245
20. Cheng *et al* (2001) Mol. Cell. 8: 705–711
21. Gong *et al* (2001) Nat. Immunol. 2: 29–36
22. Rincón (1998) J. Exp. Med. 188: 1817–1830
23. Gomez *et al*. (2001) Immunity 15: 703–713
24. Harris *et al*. (2001) J. Biol. Chem. 276: 37754–37760
25. Dong *et al*. (2000) Nature 405: 91–94
26. Dijkers *et al*. (2001) Curr. Biol. 10: 1201–1204
27. Suzuki *et al*. (2001) Immunity 14: 523–534
28. Jones *et al*. (2000) J. Exp. Med. 191: 1721–1734
29. Wang *et al*. (2001) Mol. Cell. Biol. 21: 3451–3461
30. Wang *et al*. (2001) J. Immunol. 167: 5099–5105
31. Bouillet *et al* (2000) Science 286: 1735–1738