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Dying to protect: Cell Death and the control of T cell Homeostasis

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Summary

T cells play a critical role in immune responses as they specifically recognize peptide/MHC complexes with their T cell receptors (TCRs) and initiate adaptive immune responses. While T cells are critical for performing appropriate effector functions and maintaining immune memory, they also can cause autoimmunity or neoplasia if misdirected or dysregulated. Thus, T cells must be tightly regulated from their development onward. Maintenance of appropriate T cell homeostasis is essential to promote protective immunity and limit autoimmunity and neoplasia. This review will focus on the role of cell death in maintenance of T cell homeostasis and outline novel therapeutic strategies tailored to manipulate cell death to limit T cell survival (eg autoimmunity and transplantation) or enhance T cell survival (eg vaccination, immune-deficiency).

Keywords

T Cells; Autoimmunity; Infectious Diseases; Apoptosis/Autophagy; Memory

Kinetics of T cell responses

CD4⁺ T cell responses

Naïve CD4⁺ T cells are activated when their TCRs recognize MCH-II bound peptides on host antigen-presenting cells (APCs). In individual mice, there exists between 20 to 1,000 naïve CD4⁺ T cells that have specificity for that antigen (1). CD4⁺ T cell activation leads to differentiation into multiple effector and regulatory subsets which promote or control adaptive immune responses. Distinct CD4 subsets are defined by the expression of

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transcription factors and effector cytokines and perform specialized immune functions. After the resolution of the immune response, about 90% of the activated CD4⁺ T cells die, leaving behind a population of long-lived memory cells, which on secondary challenge with the same antigen, mount a more rapid and efficient response to clear the antigen.

The mechanisms involved in the generation and maintenance of memory CD4⁺ T cells are not as well studied as those of CD8⁺ T cells, although clues are emerging. Interestingly, the markers expressed on CD4⁺ effectors are distinct from those expressed on CD8 effectors. For example, unlike CD8⁺ T cells, high expression of CD127 does not mark memory precursor CD4⁺ T cells and CD127^{low} effector CD4⁺ T cells readily re-express CD127 (2, 3). One study defined effector CD4⁺ T cells based on the expression of P-selectin ligand-1 (PSGL-1) and Ly6C into three distinct subsets, PSGL-1^{low}Ly6C^{low}, PSGL-1^{hi}Ly6C^{low} and PSGL-1^{hi}Ly6C^{hi} (3). Over time, the PSGL-1^{hi}Ly6C^{low} cells were slightly more enriched and more capable of expanding in response to a secondary challenge. They were also preferentially enriched for central memory T cells (T_{CM}) and shared a similar transcriptional profile with memory CD4⁺ T cells suggesting that this subset most likely contains memory precursors. Another study defined effector CD4⁺ subsets based on the expression of CXCR5 and PD-1 into three major subsets, CCR7⁻ Tbet^{hi}CXCR5⁻PD1⁻ (Th1), CXCR5^{hi}PD-1⁺(Tfh) and CCR7⁺ CXCR5^{int} PD-1⁻ (T_{CM})(4). While Tfh cells waned over time, Th1 cells contracted more vigorously and T_{CM} cells contracted less vigorously. Mechanisms underlying the differential contraction of these responses remain unclear. In secondary responses, T_{CM} subsets can give rise to all three subsets, while Th1 cells gave rise to only Th1 cells. An additional study defined CD4⁺ effector subsets based on the expression of Ly6C and CXCR5. Effector CD4⁺ T cells were divided into 2 major subsets, Ly6C^{hi} CXCR5⁻ Tbet^{hi} (Th1) and Ly6C^{low} CXCR5⁺ Tbet^{low} (Tfh). After memory development, three subsets were seen to persist, CXCR5⁻ Ly6C^{hi}, CXCR5⁺Ly6C^{low} and CXCR5⁺Ly6C^{int} (5). Based on their committed recall responses, the CXCR5⁻ subset was shown to be Th1 memory while the two CXCR5⁺ subsets were Tfh memory cells and T_{CM} cells were found to exist in both memory populations. The overlap and relationship between the effector CD4⁺ T cell subsets identified by these studies remain unclear and some of the conflicting results may be due to differences in the experimental model systems. The role of cell death and CD4⁺ T cell homeostasis will be discussed in section 4.

CD8⁺ T cell responses

CD8⁺ T cells are important weapons in the immune arsenal against intracellular pathogens. They produce inflammatory cytokines, such as TNF α and IFN γ to modulate the activity of multiple types of immune cells. They also elaborate cytotoxic killing to remove infected cells or cancer cells.

Recent data have shown that, to any given antigen, there exists between 20 to 1000 “naïve” CD8⁺ T cells in one mouse that have specificity for that antigen, similar to CD4⁺ T cells (6). Following antigenic encounter, these CD8⁺ T cells undergo a proliferation and begin an orchestrated process of differentiation (7). This rapid expansion phase occurs within a few days after the immune response is initiated. For example, mice infected with lymphocytic choriomeningitis virus (LCMV), the peak of CD8⁺ T cell responses is reached on day 8 to

day 10 post-infection (8). These activated effector CD8⁺ T cells are heterogeneous with divergent cell surface phenotypes and functions, which are strictly controlled by complex transcriptional networks (9). Antigen-TCR interaction, co-stimulatory signals, and inflammatory cytokines activate CD8⁺ T cells and drive the upregulation of CD44 and the downregulation of CD62L and IL-7R α (CD127). These early effector cells (EECs) also express low levels of killer cell lectin-like receptor subfamily G member 1 (KLRG1). Prior studies have shown that these KLRG1^{lo}CD127^{lo} EECs are early differentiated effector cells and have a potential to further convert into other subsets (10). One of the major subsets has been referred to as terminal effector cells or short-lived effector cells (SLECs), these cells express a KLRG1^{hi}CD127^{lo} cell surface phenotype. This population contracts rapidly after the peak of immune responses and has limited potential to develop into memory T cells (10, 11). Another major effector sub-population expresses low levels of KLRG1 and high levels of CD127 (KLRG1^{lo}CD127^{hi}) and has been referred to as pre-memory cells or memory-precursor effector cells (MPECs). Previous studies showed that this population contains the precursors of long-lived memory T cells and provides better protective immune memory (10, 11). Both populations perform effector functions in secreting inflammatory cytokines and cytotoxic activity. Another population of effector CD8⁺ T cells has a KLRG1^{hi}CD127^{hi} phenotype and has been termed double-positive effector cells (DPECs). Their function has not been well studied. However, some recent data showed that DPECs are able to produce effector cytokines and have intermediate potential to generate long-lived memory (10, 12).

Following the peak of the response, 8–10 days post-infection in the LCMV model for example, the CD8⁺ T cell population rapidly contracts in size. Roughly 80–90% of activated antigen-specific CD8⁺ T cells die via apoptosis (8), while a small fraction of them can remain for years as long-lived memory CD8⁺ T cells, and provide the basis for protective immune memory. The memory cells possess two major subsets, central memory T (T_{CM}) cells, characterized largely by expression of CD62L and CCR7, and effector memory T (T_{EM}) cells, characterized largely by the lack of CD62L expression (13–15). Both subsets are derived largely from the pre-memory cells (MPEC), although recent work suggests that the double positive effector cells (DPEC) may give rise to T_{EM} cells (16, 17). In addition, a recently described subset of tissue resident memory cells has been described. These T_{RM} cells reside in tissues, although the origin of these cells (whether they are derived from MPEC, DPEC, or SLEC) remains unclear (13, 18). Nonetheless, when a secondary immune challenge is initiated by the same antigen, these memory CD8⁺ T cells rapidly perform their immune functions and expand the population size to clear the antigen (14). As T_{CM} cells are thought to be retained in the secondary lymphoid organs, they are thought to be a renewable source of effector T cells, while the T_{EM} and T_{RM} cells are thought to be a first line of defense against secondary infection, given their blood and tissue residence. The apoptotic mechanisms which control CD8⁺ T cell homeostasis will be discussed in Section 4.

Mechanisms of Cell Death

Apoptosis

Apoptosis is the primary mechanism of programmed cell death of development and homeostasis throughout the lifetime of an organism (19). Not only is it the mechanism by

which tissue is sculpted during embryonic development (20) and by which cell proliferation is offset during homeostatic cellular turnover (21), but it is crucial to preventing pathogenesis by eliminating cells which have become irrevocably damaged (21), virally infected (22), potentially malignant (23, 24), or lymphocytes which are self-reactive (25, 26) or must be culled following the clearance of antigen (27). Indeed, the appropriate elimination of lymphocytes by apoptosis is indispensable following the explosive clonal expansion of T cells during an immune response. Following the elimination of the antigen driving the response, the majority of the expanded T cells die via apoptosis, leaving a small number to become memory cells. This winnowing of the augmented T cell population serves not only to prevent the unnecessary energy expenditure of sustaining this population, but is also a crucial mechanism to reduce lymphadenopathy or autoimmunity (28). These processes will be discussed in more depth in the upcoming section.

Apoptosis is initiated by two main pathways: the extrinsic pathway, induced by ligation of death receptors (DRs) on the cell surface; and the intrinsic pathway, which is controlled by the balance between pro-apoptotic and anti-apoptotic Bcl-2 family members (29). Both pathways converge on the activation of members of the cysteine protease family called caspases, which are present in the cell in zymogen form (30–33). The extrinsic pathway is triggered when a transmembrane DR (such as TNFR, Fas, TNFR-1, TRAIL R1, and TRAIL R2) undergoes trimerization after binding its ligand, which initiates receptor clustering and recruitment of adaptor proteins to the intracellular domain of the DR in order to form the death-inducing signaling complex (DISC) (34–36). In turn, initiator caspases (such as pro-caspase-8) are recruited to the DISC via their death-effector domains (DEDs) or caspase recruitment domains (CARDs) and undergo auto-proteolytic cleavage into their active forms (37–40). Upon activation, the initiator caspases can cleave effector caspases (i.e. caspase-3, -6, or -7), which will then initiate the cascade of the proteolytic intracellular disintegration (39).

The intrinsic, or mitochondrial, pathway, is controlled by the balance of Bcl-2 family members, which consist of pro-apoptotic (Bim, Bax, Bak, Puma, Noxa, Bad, Bcl-Xs, Bid, Bik) and anti-apoptotic (Bcl-2, Mcl-1, A1, Bcl-XL, Bcl-W) members (28). These pro- and anti-apoptotic mediators function by physical interaction, which suggests that levels of these proteins are critical for their function (41, 42). Although initial results suggested a rather simplistic model in which an overall balance between pro- and anti-apoptotic Bcl-2 family members is critical for cell survival, our and others' recent data suggest that specific interactions between Bcl-2 family members is critical for cell fate (43–45). Various cellular insults increase the expression of particular pro-apoptotic Bcl-2 family members. For example, the anti-apoptotic members keep the so-called messenger pro-apoptotic members (i.e. Bim, Bad, Puma, and Noxa) bound and sequestered from signaling to the downstream 'effector' pro-apoptotic members (i.e. Bax and Bak). The occurrence of overwhelming cell stress, hypoxia, oxidative stress, or irreparable DNA damage causes the upregulation of expression or the redistribution of these molecules to the mitochondria (46). Once these downstream pro-apoptotic members are activated, they create pores in the outer membrane of the mitochondria, allowing cytochrome c to be released into the cytosol, where it forms a complex with apoptotic protease activating factor 1 (APAF-1) and pro-caspase-9 called the apoptosome (39, 47). Pro-caspase-9 is then activated, and subsequently activates the

downstream effector caspase-3 (39). It is upon this activation of caspase-3 that the extrinsic and intrinsic pathways converge. When caspase-3 activation occurs, a cascade of events is initiated, the end result of which is the degradation of structural and regulatory molecules in the cell; the intranuclear lamina, cytoskeleton, mitochondria, endoplasmic reticulum, and DNA are all targeted for degradation (48). This results in the morphological changes associated with apoptosis: DNA condensation and degeneration, cytoplasmic condensation, and the release of apoptotic 'blebs' containing fragments of DNA and organelles (21). The release of chemoattractants and the externalization of engulfment receptors such as phosphatidylserine ensure that the apoptotic cell is readily detected and removed by phagocytes in an efficient and non-inflammatory manner which is vital to the maintenance of homeostasis and immune tolerance (49, 50).

Autophagy

Autophagy is the process wherein intracellular components are enveloped in a double membrane known as the autophagosome and degraded upon lysosomal fusion (51). This is a critical homeostatic process to recycle old or damaged organelles, providing efficient turnover of cellular contents, and also functions to protect the cell; damaged mitochondria, for instance, can release harmful reactive oxygen species (ROS) into the cell (52, 53). Autophagy also functions as a survival mechanism in times of starvation; non-essential cellular components can be broken down as a temporary solution to nutrient deprivation (54). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (55). Macroautophagy is the process by which cytoplasmic contents are enveloped by a double membrane known as the phagophore and transported to the lysosome to undergo lysosomal degradation, and is the process usually indicated by the general term "autophagy" unless otherwise specified (51). Microautophagy refers to the process by which the lysosome itself endocytoses small portions of the cytoplasm and degrades them, though its physiological relevance in mammalian cells remains poorly understood (55). Chaperone-mediated autophagy occurs when KFERQ-like pentapeptides on cytoplasmic proteins are recognized by the chaperone protein heat shock cognate 70 (Hsc70), which along with co-chaperones facilitates their translocation across the lysosomal membrane using the transmembrane protein Lamp-2A as a receptor (56–58).

Macroautophagy consists of three phases: sequestration, degradation, and amino acid/peptide generation (59). Sequestration is initiated with the formation of the isolation membrane, or phagophore, a flat double-membrane structure that engulfs cytoplasmic contents, including organelles, by elongation until it fully encloses its contents into an autophagosome (59). This process is governed by autophagy-related genes (ATG) and their corresponding proteins, originally identified in yeast (60, 61). The so-called "core" Atg proteins are highly conserved, and function similarly in mammalian cells (61). Beclin-1, the mammalian ortholog of Atg6, is the best-studied of these proteins in mammals (62). The binding partner of Beclin-1 is the anti-apoptotic protein Bcl-2; this interaction is decreased in times of nutrient deprivation, freeing Beclin-1 to mediate the formation of the autophagosome (63–65). Once the isolation membrane has been formed around the cytoplasmic contents, the autophagosome fuses with a lysosome and its contents are

degraded by the lysosomal hydrolases (66). The broken down contents can then be exported into the cytoplasm for reuse by the cell (67).

Paradoxically, despite being recognized as a survival mechanism in cells, autophagy may also be a mechanism of programmed cell death. Cells that have undergone autophagic cell death are characterized by their abundant phagosomes and self-degradation by their own lysosomes rather than phagocytic destruction (68). Although whether these cells died due to autophagy or simply had ongoing autophagy when they died is still controversial (69), there are studies indicating that autophagy is in fact a mechanism of programmed cell death. Mouse embryonic fibroblasts (MEFs) with both Bax and Bak knocked out will undergo cell death that is not consistent with apoptosis, but which instead is characterized by increased numbers of autophagosomes and autolysosomes and can be blocked by Beclin-1 or Atg5 knockdown (70). Additionally, a study by Liu et al. has shown that autophagic cell death can be induced by the autophagy-inducing peptide Tat-Beclin-1, and that this death can be inhibited by inhibition of autophagy, not by inhibition of apoptosis or other mechanisms of programmed cell death (71). Autophagic cell death appears to be an important mechanism of cell death during development, as research in *Drosophila* development has shown that deficiency in Atg proteins, but not caspases, prevents death of larval midgut cells in the intestine (72).

The mechanism(s) by which autophagic death is accomplished, however, is not completely understood. It has been postulated that cell death by autophagy could result simply from the degradation of the bulk of cellular contents or from the more targeted destruction of proteins crucial to cell survival (73). Yu et al. (2006) found that inhibition of apoptosis by caspase-8 inhibition results in cell death subsequent to the degradation of a key cellular antioxidant, catalase, causing the accrual of substantial amounts of reactive oxygen species (ROS), which in turn resulted in membrane peroxidation and loss of integrity (74). There is also evidence that autophagy contributes to cell death by degrading the *Drosophila* inhibitor of apoptosis (IAP) protein dBruce, leading to caspase activation and DNA fragmentation and triggering programmed cell death (75).

Furthermore, evidence also exists for a shared set of proteins and extensive crosstalk between the autophagic and apoptotic pathways. An important mechanism by which this cross-regulation occurs is through the interaction between Beclin-1 and Bcl-2. Beclin-1 is sequestered in the cell by Bcl-2 during non-starvation conditions, and can also interact with, and be inhibited by, other anti-apoptotic members of the Bcl-2 family through its BH3 domain (64, 65). Beclin-1 in human ovarian surface epithelial cells with induced expression of H-Ras, for instance, can be inhibited by Bcl-2, Bcl-xl, and Mcl-1 (76). The presence of the pro-apoptotic protein Noxa, however, will displace Mcl-1 from Beclin-1, likely due to its higher affinity for Mcl-1, freeing Beclin-1 to initiate autophagy and caspase-independent autophagic cell death (76). In addition to regulation by Bcl-2 family members, autophagy can also be modulated by pro-apoptotic proteases; Beclin-1 and Atg5 can be cleaved by caspases and calpains, respectively, which converts them into pro-apoptotic proteins which mediate the release of cytochrome c from the mitochondria (77, 78). The contradictory role of autophagy in driving both cell survival as well as death has made it difficult to fully understand the mechanisms underlying autophagic cell death.

Necroptosis

Necroptosis, or programmed necrosis, is a mechanism of cell death that shares some morphological features with necrosis, which is generally considered an uncontrolled form of cell death due to injury, but is the result of a regulated signaling cascade (79). Necrotic cell death, both programmed and accidental, is characterized primarily by the swelling of organelles and oncosis, an increase in cell volume, followed by cell lysis and the disintegration of the plasma membrane (80). In contrast to the efficient and immunologically silent removal of apoptotic cells, necroptosis is an inflammatory process, releasing danger-associated molecular patterns (or DAMPs) upon cell lysis (81).

Necroptosis can be induced upon ligation of death receptors (TNF receptor 1 (TNFR1), CD95 (Fas), and TRAIL-R1/R2 have been linked to necroptosis stimulation) as well as through stimulation of damage and infection sensing receptors such as Toll-like receptors (TLRs) 3 and 4 and the cytosolic sensor DNA-dependent activator of IFN regulatory factors (DAI) (79, 82, 83). Ligation of these receptors of course is more commonly associated with inflammation and cell survival (in the case of TNFR1, TLR3/4, and DAI) or the induction of apoptosis (upon FasL or TRAIL binding), but is highly context-dependent and the resulting signaling pathways can also result in necroptosis in certain circumstances (79, 82, 83). Whether signaling through these receptors results in necroptosis is dependent upon serine/threonine kinase receptor-interacting protein 1 (RIPK1), RIPK3, and caspase-8 (79, 84–86). TNF binding, for instance, can result in the protein complex composed of TNFR1-associated death domain protein (TRADD), RIPK1, and cellular inhibitors of apoptosis (cIAP1/2), among others, that induces the signaling cascade that activates NF- κ B and AP-1 (Complex I), resulting in proinflammatory signals (87, 88). However, if Complex I is destabilized, TRADD binds Fas-associated protein with death domain (FADD) and caspase-8, forming Complex IIa (88). The active caspase-8 in Complex IIa promotes apoptosis and simultaneously negatively regulates necroptosis by cleaving RIPK1 and RIPK3 (89–91). However, upon the inhibition of IAPs or certain members of Complex I, this leads to the formation of Complex IIb, which consists of RIPK1, RIPK3, FADD, cFLIP, and caspase-8 (92, 93). RIPK1 and RIPK3, through interactions of their RIP homotypic interaction motifs (RHIMs) (84, 85), form a large amyloid-like structure known as the necrosome (94), which is essential to the recruitment and phosphorylation of the downstream effector protein of necroptosis, mixed-lineage kinase domain-like protein (MLKL) (95, 96). MLKL will then oligomerize, transport to the cell membrane, and mediate membrane permeabilization, leading to the release of DAMPs and the death of the cell (97–102). This release of DAMPs can activate Toll-like receptors (TLRs) of surrounding cells, activating NF- κ B and resulting in the release of more TNF- α , increasing and perpetuating the inflammatory response and potentially leading to necroptosis of surrounding cells.

Necroptosis appears to function as an alternative, backup mechanism to apoptosis, and may have evolved due to the ability of a myriad of viruses to circumvent apoptosis by inhibiting caspase-8 (103, 104). For instance, mice infected with vaccinia virus, which encodes a caspase inhibitor, undergo necroptosis, while mice deficient in RIPK3 are highly susceptible to vaccinia infection (84). This “failsafe” role for necroptosis is further supported by data showing that the deletion of caspase-8 in T cells is able to restrain the lethal autoimmunity

caused by the deletion of Bim via the activation-induced necroptosis of the autoreactive T cells (105). The autoreactive T cells which escaped negative thymic selection due to the loss of Bim were largely eliminated when caspase-8 was not present to negatively regulate necroptosis, thereby suppressing the autoimmunity normally exhibited in Bim^{-/-} mice (105). The role of necroptosis in T cell homeostasis under normal conditions, however, is controversial; given mouse strain-specific differences in the ability of necroptosis to regulate T cells (106) and patients with caspase-8 mutations who exhibit lymphoproliferative disease indicative of a lack of compensatory necroptosis (107), much still remains to be elucidated about the role of necroptosis in T cell homeostasis.

Pyroptosis

Pyroptosis is another form of programmed necrosis which is mediated by one or more inflammatory caspases (caspase-1 and -11 in mice, -1, -4, and -5 in humans) following inflammasome activation (108–110). It shares the morphological characteristics of other forms of necrosis: the swelling of the cytoplasm and cell and the formation of pores in the plasma membrane followed by rupture of the cell (109, 111). The word pyroptosis, deriving from the Greek “pyro,” meaning fire, and “ptosis” (“falling”), denoting it as a mechanism of programmed cell death, refers to the inflammatory nature of this process; both DAMPs and proinflammatory cytokines such as IL-1b and IL-18 are released upon cell lysis, provoking a potent inflammatory response (109, 112).

The inflammasome, which is activated upon stimulation of pathogen recognition receptors (PRRs) such as TLRs and NOD-like receptors (NLRs), is a large multimeric protein complex that is required for the cleavage of pro-caspase-1 into its active form, which is in turn required for cleavage of the leaderless cytokines IL-1b and IL-18 (113). The inflammasome consists of a sensor protein, commonly a member of the NLR family of proteins (such as NLRP1, NLRC4, NLRP3, or NLRP6) or AIM2-like receptors, and can also contain the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC) (113–115). A CARD-domain containing protein in the complex (ASC, NLRP1, NLRC4) is able to recruit and activate caspase-1 (113). Activation of the inflammasome can be induced upon the recognition of a variety of pathogen-associated molecular patterns (PAMPs) or other cellular stressors by the sensor protein, which triggers caspase-1 mediated cleavage of the pro-inflammatory cytokines IL-1b, IL-18, and can induce pyroptosis (116–118). The effector molecule mediating pyroptotic cell death was only recently identified as gasdermin D, which is structurally autoinhibited and must be cleaved by caspase-1 or -11 (119, 120). The N-terminal domain of the protein (gasdermin-N) then translocates to the cell membrane where it binds to phosphatidylinositides and oligomerizes to form pores in the membrane, disrupting membrane potential and causing cell lysis (120–123).

Pyroptosis acts as an important host defense mechanism, not only by its release of pro-inflammatory cytokines and DAMPs, but also as a method of depriving intracellular pathogens of their host cell and replicative niche, externalizing pathogens for neutrophilic clearance (124). Indeed, although caspase-1 has the ability to perform all of these functions, caspase-11 is an inefficient processor of IL-1b and IL-18, and its function in this pathway appears to be primarily to mediate pyroptotic cell death, as evidenced by its importance in

clearing cytosolic bacteria such as *Burkholderia* (124). Interestingly, this defense mechanism may not always be to the advantage of the host, as it may play a role in HIV pathogenesis; when CD4⁺ T cells are abortively infected with HIV, viral DNA transcripts accumulate in the cytosol and are detected by intracellular DNA sensors (125, 126). This results in inflammasome assembly and pyroptosis, the resulting release of cytokines and DAMPs creating a feed-forward loop of inflammation, pyroptosis in surrounding cells, and increased loss of CD4⁺ T cells (127). However, the specific role of pyroptosis in controlling naïve, effector and memory T cell homeostasis remains unclear.

Bcl-2 family members

Although the extrinsic, or death receptor regulated pathway, contributes to T cell homeostasis (128, 129), the intrinsic, or Bcl-2 regulated pathway plays a more substantial role in controlling normal T cell homeostasis. As mentioned previously, the Bcl-2 family consists of pro- and anti-apoptotic members that physically interact with each other to regulate apoptosis. Recent work is starting to reveal a substantial amount of specificity between Bcl-2 family members that regulate their ability to drive apoptosis, especially in T cells. In this section, we examine the major individual Bcl-2 family members, their role in T cell homeostasis and, if known, discuss the experimental data that shows their specificity for other family members.

Bim

Bim is a pro-apoptotic BH3-only molecule which critically controls T cell development and homeostasis (7, 28, 130). Bim is encoded by *Bcl2l1* gene and has three major splicing isoforms, Bim_S, Bim_L, and Bim_{EL} (131). In T cells, the more than 90% of Bim protein exist as Bim_{EL}, the remainder is Bim_L, and little if any Bim_S (132). Of the pro-apoptotic BH3-only Bcl-2 family members, Bim appears to play a major and largely non-redundant role in controlling T cell homeostasis.

Bim plays a substantial role in shaping T cell development in the thymus. Initial work suggested that Bim is critical for the apoptotic elimination of self-reactive thymocytes (133). However, this work is somewhat controversial as others have not observed a significant role for Bim in other models of thymocyte negative selection (134–136). In subsequent work, Puma appears to have redundancy with Bim in controlling the thymic elimination of self-reactive thymocytes (137). In addition, our and others recent data suggest a major role for Bim in controlling the death of agonist-selected thymocytes (e.g Foxp3⁺ Tregs, precursors of CD8αα⁺ intestinal intraepithelial lymphocytes (IELs), and NKT cells) (138–141). Agonist selected thymocytes appear to be on the higher end of the avidity spectrum, and although it was once thought that they avoided negative selection, it appears that there is a substantial amount of Bim-dependent culling of agonist-selected thymocytes. This culling plays an important role in limiting the numbers of cells that adopt this developmental fate.

In the periphery, the homeostasis of both naïve CD4⁺ and CD8⁺ T cells is also controlled by Bim (44, 133). Further, our and others' data showed that Bim is the major mediator of T cell contraction after the peak of immune responses. T cell-specific loss of Bim significantly prolongs the survival of activated antigen-specific CD4⁺ and CD8⁺ T cells (44, 132, 142–

146). In CD8⁺ T cells, Bim drives the contraction of both KLRG1^{lo}CD127^{hi} MPEC and KLRG1^{hi}CD127^{lo} SLEC (143). These data show that Bim not only culls terminal effector cells but also severely limits the cells available to enter the memory compartment.

One major question is whether or not this accumulation of memory T cells in Bim-deficient mice are functional? We and others have shown that deficiency in Bim results in increased both effector and memory CD4⁺ T cells during infection (147, 148). We showed that, in vitro, Bim-deficient T cells responded to antigen with as much if not more cytokine production relative to cells from control mice (147). Moreover, we showed that Bim limits the persistence of IFN γ -producing CD4 effector T cells in a model of *Leishmania major* chronic infection. Further, Bim^{-/-} mice have stronger CD4⁺ T cell responses and substantially increased clearance of *L. major* parasites (2–3 log decrease) after secondary challenge, showing that Bim critically dampens CD4 T cell-mediated immune memory (147). Further, the absence of Bim circumvented the requirement for antigen-persistence to maintain memory in this model as most Bim-deficient mice cleared the initial infection, but maintained substantial protective immunity to re-challenge. Thus, Bim clearly limits the memory compartment, suggesting that interference with Bim could be a novel therapeutic approach to enhance protective immunity.

Bim also critically controls the homeostasis of CD4⁺Foxp3⁺ Treg cells. As Treg are thought to be an agonist-selected population, Bim plays a substantial role in culling Treg during thymic development (138, 141). Indeed, we showed that Bim appears to function at the cortex or cortico-medullary junction to drive the death of Treg precursor cells (138). In addition to its role in controlling thymic Treg development, we found that Bim is a major regulator of peripheral Treg homeostasis. We and others have shown that Treg accumulate substantially in aged mice; roughly 35% of CD4⁺ T cells in 18 month old mice are Foxp3⁺ (149–152). Bim-deficient mice reach this high level Treg accumulation as early as 6 months of age (149, 153, 154). Further, this role of Bim is intrinsic to Treg as FoxP3-Cre-Bim^{f/f} mice have a similar accumulation as mice globally deficient in Bim (154). This Treg accumulation with age was largely due to the effects of Bim on peripheral survival, rather than increased thymic output or peripheral conversion (149). Bim was particularly efficient in limiting the numbers of CD44^{hi} CD62L^{lo} “effector” Tregs (153).

As Bim is a central mediator of T cell homeostasis in various contexts, its expression and function is tightly regulated. Indeed, the expression of the *Bcl2l1* gene is regulated by multiple mechanisms, including epigenetic, transcriptional, and post-transcriptional, levels. Furthermore, the function of Bim is regulated by post-translational modifications (e.g. phosphorylation) as well as by interactions with anti-apoptotic partners. Previous studies showed that Bim is epigenetically controlled in several cell types (155–159). Our unpublished data further suggest that DNA methylation represses Bim expression in CD8⁺ T cells (K-P.L and D.A.H), although this mechanism appears not to require DNA methyltransferase 3a (160). Multiple transcription factors appear to regulate the expression of Bim, including Foxo3a, E2F1, E2A, and Irf4 (161–166). The signals and situations in which these TFs control expression of Bim appear to vary. For example, Foxo3a may function to enhance expression of Bim when cytokine-driven Akt signaling diminishes and fails to inactivate and restrain Foxo3a from interacting with the Bim promoter (163, 164). In

CD8⁺ T cells, Irf4 directly binds to *Bcl2l1* locus and represses Bim levels (162). However, Bim expression in Irf4^{-/-} CD4⁺ T cells remain the same as WT CD4⁺ T cells, which suggests a cell-specific mechanism (167). Transcription factor E2F1 also directly promotes Bim expression and mediates TCR-activation-induced cell death (165, 168). Id2 was recently described to repress expression of Bim within antigen-specific CD8 effector cells after *Listeria* infection (169), likely through inhibiting E2A-mediated transcription of Bim (166, 170). However, the additional loss of Bim failed to rescue Id2-deficient terminal effector cells (166). Further, prior reported increases in Bim in Id2-deficient T cells could be related to their accumulation of pre-memory cells, which we previously showed have increased levels of Bim (142). Thus, the factors that control expression of Bim within activated T cells remain unclear.

Bim is also regulated by post-transcriptional mechanisms. The 3'-UTR of Bim mRNA transcript is targeted by the miR-17-92 microRNA cluster, which post-transcriptionally limits Bim levels (171–173). miR-24 and miR-148a were also shown to post-transcriptionally repress Bim levels (174, 175). In addition, Bim proteins are post-translational controlled by ERK and JNK (176, 177) as well as via interactions with anti-apoptotic Bcl-2 family members (28). Although several mechanisms have been described to regulate the expression/function of Bim in other cell types, more work is required to determine the specific mechanisms involved in the control of Bim expression and function within naïve, effector, and memory T cells.

Bcl-2

Bcl-2 is an anti-apoptotic member of Bcl-2 family, originally identified in follicular B cell lymphoma as a proto-oncogene (178–180). The Bcl-2 gene has an unusual structure with just two exons and an enormous intron, spanning over 370 Kb in human genome (178, 181–183). This locus shares some homology with the Ig locus as one of the initial discovery of Bcl-2 as a proto-oncogene showed that Bcl-2 translocated to the Ig locus and this Bcl-2 overexpression promoted B cell lymphoma development (180). Two groups at the same institution generated Bcl-2-deficient mice (184, 185). Both groups found that genetic ablation of Bcl-2 resulted in mice with polycystic kidney like disease which was lethal by 5 weeks of age. Further, Bcl-2-deficient mice displayed premature greying of the fur and a dramatic loss of thymocytes and lymphocytes (184–186).

Bcl-2 is critical for maintaining naïve and memory T cell survival (44, 184, 185) as well as NK cells (187), but does not appear to be required for FoxP3⁺ Treg (188). Notably, the additional loss of Bim not only rescued survival of the entire animal (189), but also rescued Bcl-2-deficient naïve and memory T cells (44, 132, 134, 142, 190–192). In effector CD8⁺ T cells, we and others found that SLEC had significantly reduced levels of Bcl-2, while MPEC maintained higher expression of Bcl-2 (142). Interestingly, Bim levels tracked with Bcl-2, with MPEC cells having significantly higher levels of Bim (142). Again, genetic and pharmacologic disruption of Bcl-2 function dramatically reduced MPEC survival. In fact, the only MPEC cells that were allowed to survive in the absence of Bcl-2 were cells that had extremely low levels of Bim (142). Finally, to examine the normal expression of Bim and Bcl-2 in cells that would otherwise die by a Bim-driven death, we removed the ability of

Bim to kill T cells by the T cell specific loss of downstream Bax in mice that were also deficient in Bak. Interestingly, in the absence of Bax and Bak, Bim and Bcl-2 levels were uncoupled and cells having high levels of Bim and low levels of Bcl-2 were observed (142). Thus, Bcl-2 plays an obligatory role in combating Bim to promote CD8⁺ T cell memory development.

The expression of Bcl-2 is repressed by cytokine deprivation and reactive oxygen species (ROS) (193–196) transduction and productive T cell responses, but also represses Bcl-2 expression and eventually promotes the demise of T cells (195, 197–199). In addition, the process of T cell expansion limits cytokine availability as the T cell population grows exponentially following antigenic stimulation. This cytokine withdrawal significantly reduces Bcl-2 expression as we showed that IL-7 and IL-15 maintain Bcl-2 and effector CD8⁺ T cell survival through a Stat5-dependent pathway (2, 190, 200). Interestingly, we found that neither STAT5, nor Bcl-2 was strictly required for effector CD4⁺ T cell survival (2, 190). Thus, the anti-apoptotic factors that control CD4⁺ T cell survival remain unclear.

A1 (Bfl-1)

A1 is an anti-apoptotic Bcl-2 family member, originally identified as a granulocyte and macrophage colony stimulating factor early response gene (201). Mouse *Bcl2a1* has four copies (*A1-a*, *A1-b*, *A1-c*, *A1-d*) encoded on chromosome 9 with *A1-c* being a pseudogene (202). Mouse A1 is expressed predominantly in hematopoietic cells and embryonic tissues (203, 204). In contrast, humans and rats express only one copy of A1, called Bfl-1 (Bcl-2-related gene expressed in fetal liver) with a homology of about 72% to mouse A1. Bfl-1, is located on chromosome 15 in humans and is expressed in both hematopoietic and non-hematopoietic cells (205, 206). Another gene called the Glasgow rearranged sequence which differs from Bfl-1 only by 2 amino acids was identified in multiple cancer lines (207).

The regulation of A1 in T cells mainly occurs at the transcriptional level. The main trigger for A1 expression is TCR signaling whereas common gamma chain cytokines do not increase A1 transcripts (208, 209). Pre-TCR signaling in the thymus also upregulates A1 (210). Mechanistically, NF- κ B, calcium-dependent signals (208) and MEK1/2 signals (211) have shown to be required for TCR-driven A1 induction, while the repressive transcription factor, Blimp-1, is reported to antagonize A1 expression (212). Co-stimulation via 4-1BB (213) and OX40 (214, 215) leads to induction of A1 while CD28 has no effect (208). Not much is known about the post-translational regulation of A1, except one report that A1 binding to Bim_{EL} improves its stability (216). A1 has a very short half-life (216), but an E3 ligase that mediates its proteosomal degradation is yet to be discovered.

A1 is known to interact with other pro-apoptotic Bcl-2 family members. A1 binds strongly to Bim, Bid, Puma and with weaker affinity to Noxa (217) and displays no affinity for Bad, suggesting a similar binding profile as Mcl-1. Reports of A1 binding to the multi-domain Bax and Bak are controversial. However, much of these studies have relied on in vitro systems, thus, the A1 binding partners in T cells have yet to be verified in physiological settings. The role of A1 in T cell development and homeostasis is in the nascent stages of exploration. During T cell development, A1 is expressed upon pre-TCR signaling during beta selection (210, 218). A1 has been postulated to be important for viability of DP

thymocytes awaiting positive selection, but does not appear to affect negative selection (219). Transgenic overexpression of A1-a led to an increase in DP thymocytes and partial protection from pro-apoptotic stimuli (209). However, RNAi experiments targeting A1 yielded varying results. One group showed a significant loss of DP thymocytes while another group showed no impact on thymocyte development and attributed this to either inefficient RNAi or a minimal/redundant role for A1 in T cell development (220, 221). Additionally, no enhancement of T cell apoptosis was observed in A1-a^{-/-} mice (222). This is likely explained by redundancy among the A1 genes due to high homology (>95%) or the poor expression of A1-a in T cells (208, 210). Recently, a full A1 KO mouse was created (223). Interestingly, the mouse was viable, unlike deletion of other anti-apoptotic Bcl-2 family members. Strikingly, these mice had no thymic phenotype, showing that A1-a-b-d are not necessary for thymocyte survival.

A1 does not seem to play a role in naïve T cell survival except for one report of RXR agonists mediating naïve CD4⁺ T cell survival through induction of A1 (224). However, recent work examining a complete A1 KO mouse showed no decrease in the overall numbers of naïve T cells (225). In activated T cells, A1 is mainly thought to be critical for activated T cell survival due to its rapid response to TCR signaling and the fact that it does not inhibit T cell proliferation (208, 209). Preliminary data from our lab shows higher A1 expression in CD4 effector cells compared to CD8 effectors at the peak of an anti-viral response, suggesting a CD4-specific role for A1 in effector T cell survival. Although the recent work in the complete A1 KO suggested that effector T cell responses were normal, it should be noted that this work did not examine the CD4⁺ T cell response using MHC tetramers, but instead just looked at overall numbers of memory cells after infection (225). In addition, the mice did display a significant loss of memory CD4⁺ T cells, suggesting that either the contraction of the effector response or the maintenance of the memory CD4 compartment relies substantially on A1. Interestingly, A1 responds to increasing antigenic signal strength in CD8 Tg OT-1 T cells *in vitro* (211), suggesting that A1 may promote the selection of high affinity TCR clones during clonal expansion (226). However, further work is required using the complete A1 KO animals to determine the role of A1 in the control of effector and memory T cell homeostasis *in vivo*.

A1/Bfl-1 is also differentially expressed in disease settings and could be an attractive therapeutic target. A1 inhibition could potentially prevent transplant rejections or GVHD upon bone marrow transplantation, as A1 was shown to confer resistance to allo-reactive T cells during treatment with the BH3 mimetic, ABT-737 (227). Transgenic overexpression of A1 was found to alleviate collagen induced arthritis by having an inhibitory effect on Th17 differentiation (228). A1(Bfl-1) is up-regulated in both human and mouse pre-T cell leukemias, suggesting that pre-TCR-induced A1 overexpression can contribute to T cell leukemia (210). Silencing of Bfl-1 increased etoposide-induced caspase-dependent apoptosis in Adult-T cell leukemia (229). While A1 is an attractive target in some circumstances, the potential role of in antimicrobial T cell responses could impair adaptive immunity, especially to chronic or persisting infections (230, 231). More studies in the complete A1 KO mice along with the development of A1 inhibitors will help elucidate A1's role in T cell biology and may uncover potential therapeutic applications.

Bcl-xL

Bcl-xL is another anti-apoptotic Bcl-2 family member encoded by *Bcl2l1* genes, which contains two splicing isoforms, Bcl-xS and Bcl-xL (232, 233). This gene was first identified as a Bcl-2 homologues which promotes cell survival (232). The anti-apoptotic isoform, Bcl-xL, contains highly a conserved structure to Bcl-2 and protects cells from intrinsic pathway-mediated apoptosis. For example, Bcl-xL sequesters BH3-only pro-apoptotic molecules, Bim, Noxa, and Bad, and prevents the activation of downstream Bax and Bak (234–236). In addition, Bcl-xL directly binds to Bax to prevent Bax-oligomerization on mitochondrial outer membrane, which initiates apoptosis (237). Another anti-apoptotic mechanism of Bcl-xL is through sequestering p53 from interaction with Bax (238).

In terms of T cell homeostasis, Bcl-xL regulates thymic development. DP thymocytes express high levels of Bcl-xL which supports their survival (239, 240), while these cells have a limited amount of Bcl-2 to antagonize pro-apoptotic molecules (241). In peripheral T cells, anti-CD3 stimulation drives upregulation of Bcl-xL (233) and Bcl-xL was initially described as a predominant survival factor for activated T cells (232). However, genetic KO approaches were lacking to crucially evaluate the role of Bcl-xL. More recent work using defined T cell specific loss of Bcl-xL demonstrated that Bcl-xL is not necessary for the survival and homeostasis of antigen-specific CD4⁺ or CD8⁺ T cells after infection with (240, 242) required for Treg cell homeostasis (188). These data suggest that Bcl-xL is either not involved or is partially redundant with other anti-apoptotic molecules, such as Bcl-2 or Mcl-1, in promoting peripheral T cell survival.

The expression of Bcl-xL is induced by TCR signaling and further enhanced by CD28, 4-1BB, and OX40 co-stimulation (213, 233, 243, 244), which is mediated by NF- κ B signaling (213, 245). The expression of Bcl-xL is also promoted by phosphatidylinositol 3-kinase (PI3K) signaling, which is repressed by PD-1 signaling (246). This may partially explain the mechanism of anti-PD-1 checkpoint blockade cancer therapy in promoting T cell responses.

Mcl-1

Mcl-1 is an anti-apoptotic Bcl-2 family member, originally identified as an early-induced gene during the differentiation of ML-1 human myeloblastic leukemia cells (247). Mcl-1 promotes the survival of many cell types and deletion of *Mcl-1* results in peri-implantation embryonic lethality (248). There are 2 murine Mcl-1 isoforms, one with an anti-apoptotic function, located on the outer mitochondrial membrane (249, 250) and another which supports normal mitochondrial function, found in the mitochondrial matrix (251). In humans, Mcl-1 alternative splicing results in a longer variant (Mcl-1L) which is anti-apoptotic and a smaller variant (MCL-1S), which surprisingly, acts as a pro-apoptotic BH3-only protein capable of dimerizing with the anti-apoptotic MCL-1L (252, 253). This section focuses on the anti-apoptotic role of Mcl-1 in T cells.

The regulation of Mcl-1 expression is highly complex. There is minimal transcriptional control, although one study showed that loss of specific DNA promoter elements in Mcl-1 led to tissue specific and CD4 vs CD8 lineage specific expression (254). Mcl-1 is tightly

regulated at the post transcriptional and post-translational levels. Mcl-1 has a very short half-life and is subject to ubiquitin-dependent and ubiquitin-independent degradation (255, 256). This short half-life is physiologically significant, as a stabilized form of Mcl-1 ameliorated the polycystic kidney disease and lymphopenia arising in mice lacking pro-survival Bcl-2 (257). Common gamma chain cytokines induce Mcl-1 in resting T cells *in vitro*, independent of mRNA levels and protein stability, suggesting translational regulation (258). Mcl-1 protein stability is controlled by T cell receptor (TCR) affinity-dependent interleukin-2 signaling (259). Growth factor withdrawal via GSK3 induction leads to Mcl-1 destabilization(260), however GSK3 independent degradation of Mcl-1 can also occur upon glucose deprivation(261). The interaction of Mcl-1 with other Bcl-2 family members also contributes to its post translational regulation. Noxa, binds Mcl-1 with high affinity (217) and triggers its proteosomal degradation. Conversely, Bim and Puma bind Mcl-1 and stabilize it, although the binding of Puma is not sufficient to prevent degradation of Mcl-1 (262, 263). The interaction of Mcl-1 with other Bcl-2 family members varies under different physiological conditions as discussed below.

Mcl-1 plays a critical role in T cell survival at several developmental and post developmental stages. In the thymus, loss of Mcl-1 results in impaired survival of DN, DP and SP thymocytes, although Bcl-xl can compensate for Mcl-1 loss in the DP population (264, 265). Further, Mcl-1 mainly antagonizes Bak and transgenic over-expression of Bcl-2 and deletion of Bax or Bim does not rescue Mcl-1-deficient thymocytes (266). There is an intrinsic requirement for Mcl-1 in maintenance of peripheral T cells and condition deletion of Mcl-1 results in a massive loss of both CD4 and C8 T cells (264). In the periphery, *in vitro*, resting T cells upregulate Mcl-1 in response to common gamma chain cytokine signaling and under IL-4 conditions, upregulation of Bim is reported to shift the role of Mcl-1 towards Bim inhibition (258).

Mcl-1 is also critical for the survival of activated T cells (264, 265). Mcl-1 overexpression in CD8⁺ T cells also was found to regulate the differentiation of effector and memory cells in an LCMV infection model, with SLECs being promoted at the expense of MPECs and CD8⁺ T effector memory cells being enhanced at the cost of poly-functional CD8⁺ T central memory cells (267). During lymphocytic choriomeningitis virus infection, we showed that loss of Mcl-1 resulted in a massive loss of antigen-specific CD4⁺ and CD8⁺ T cells (43). Mechanistically, GSK-3 β appears to control Mcl-1 levels and contribute to activated T cell survival (268). Mcl-1 was also shown to be induced by NKG2D/PI3K signaling of activated T cells and promoted CD8 survival during the effector to memory transition (269). Whether the GSK-3 β and NKG2D mechanisms for controlling Mcl-1 are linked or independent remain unclear. In addition, Treg cells also appear to rely on Mcl-1, but not Bcl-2, for their survival. During homeostatic proliferation driven by IL-2, Treg expansion is aided by Mcl-1 induction (188). Despite its critical role in T cell survival, the pro-apoptotic factors antagonized by Mcl-1 remain elusive. In naïve T cells, data suggest that Mcl-1 antagonizes Bak, but under conditions of cytokines withdrawal, the upregulation of Bim may drive Bak-independent death of Mcl-1-deficient T cells (270). In activated T cells, Mcl-1 has been shown to be induced in a signal strength dependent manner and is involved in the selection of high affinity CD8⁺ T cell clonotypes by antagonizing Noxa (259). Similarly, in mitogen-activated T cells, glucose deprivation leads to increased Noxa, decreased levels of Mcl-1,

and apoptosis induction, implying the involvement of Mcl-1 in restraining Noxa and promoting T cell survival (271). However, it is unclear if loss of NOXA restores Mcl-1-deficient T cells. Moreover, our data clearly show that the loss of Mcl-1-deficient T cells (CD4, CD8, and Treg) is not restored by the additional loss of Bim. Thus, given the extreme cell loss that accompanies Mcl-1-deletion, our and others data strongly suggest that Mcl-1 plays a more downstream role, inhibiting Bak/Bax or potentially inhibiting multiple BH3-only proteins (43).

Mcl-1 is dysregulated in disease conditions and is an attractive therapeutic target. In chronic infection, Mcl-1 is downregulated in CD127 low antigen specific CTLs along with up-regulation of Bim and this causes apoptosis of CD8⁺ T cells (272) or hyporeactivity of CTLs, which could possibly be treated by apoptosis inhibition or IL-7 treatment (273). Conversely, Mcl-1 enhances survival in a variety of cancers including T cell lymphomas and various Mcl-1 inhibitors are being explored (274). Given the robust loss of cells anytime Mcl-1 is deleted, there are some substantial concerns about the potential cytotoxicity of Mcl-1 inhibitors. In this case, inhibitors that may only partially inhibit Mcl-1 may actually be more effective with less off-target effects. For example, one allele of Mcl-1 is sufficient to maintain peripheral T cells (264), but might sensitize tumor cells overexpressing Mcl-1 for death.

Puma and Noxa

The BH3-domain containing pro-apoptotic “activator” proteins PUMA and Noxa play diverse roles in regulating apoptosis in T cells both in homeostatic conditions as well as in the context of DNA damage (275–280). Both PUMA (p53 upregulated modulator of apoptosis) and Noxa are perhaps best known as p53 response genes transcribed in response to DNA damage induced by genotoxic drugs or irradiation (281, 282). Interestingly, though together PUMA and Noxa are thought to be responsible for the majority of the pro-apoptotic activity of p53 (281), mice deficient in PUMA, Noxa, and p21 (the other main transcriptional target of p53 responsible for mediating cell cycle arrest rather than apoptosis) do not fully replicate the phenotype of p53 knockout mice (283). Both PUMA and Noxa can also be activated independently of p53 by other transcription factors in response to other forms of cell stress; PUMA in response to growth factor and cytokine deprivation, ER stress, or exposure to glucocorticoids (279, 280, 284, 285), Noxa as a result of ER stress or hypoxia (284, 286). Thus, mechanistically, PUMA and Noxa each has specific roles as initiators of apoptosis. PUMA has the ability to bind all Bcl-2 molecules, and can directly induce mitochondrial permeabilization by freeing Bak and Bax from Bcl-2 family sequestration as well as by activating Bak and Bax directly (45, 46, 287–290). Noxa, however, is only able to bind Mcl-1 and A1 (45), though it also is able to induce the ubiquitin-mediated proteasomal degradation of Mcl-1, and may or may not be able to directly activate Bak and Bax (291–296). It is unclear if the two BH3 domains possessed by PUMA allow it to have differential interactions with pro- and anti-apoptotic Bcl-2 family members relative all of the other BH3-only Bcl-2 family members that possess a single BH3 domain. Future work will need to examine the interacting partners for PUMA and NOXA in activated T cells and how these interactions contribute to the control of T cell homeostasis.

Both PUMA and Noxa also play a role in the control of T cell expansion and contraction during an immune response. A study by Fischer et al. (2008) showed that mice lacking PUMA are unable to fully contract their expanded CD8⁺ T cell population following clearance of HSV (297), and we have shown that both PUMA and Noxa collaborate with Bim to mediate contraction of MPEC following viral clearance (143). Noxa also appears to function in a fine-tuning capacity to limit numbers of low affinity effector CD8⁺ cells by increasing the threshold necessary for the survival of activated CD8⁺ cells (298). Noxa's role in limiting the number of memory precursor cells, however, is controversial; a study by Wensveen et al. (2013) showed a role for Noxa in limiting memory T cell formation (299), although we did not see a contribution from Noxa in limiting memory precursor cells (143).

Bax and Bak

The effector molecules of apoptotic cell death, Bax and Bak, are the downstream executioners required for BH3-mediated apoptosis, forming homo-oligomers on the outer mitochondrial membrane in order to generate membrane pores, which allows the release of cytochrome c, formation of the apoptosome, and caspase activation (300, 301). The proapoptotic activity of Bax and Bak is regulated by both Bcl-2 family members and BH3-only proteins; Bcl-2 proteins keep Bax and Bak bound and sequestered, and it is only with the activation of BH3-only proteins and the dissociation of the Bcl-2 proteins that Bax and Bak will undergo conformational changes that will (in the case of the cytosolic Bax) induce their accumulation on the outer mitochondrial membrane and cause both Bax and Bak to oligomerize (302–309). Certain members of Bcl-2 family and BH3-only proteins have been shown to play more significant roles in Bax/Bak regulation than others; BH3-only proteins Bim, PUMA, and truncated BID (tBID) appear to have a predominant role in Bax/Bak activation, as mice deficient in all three proteins exhibit extensive resistance to multiple inducers of apoptosis (290). Bcl-2 is critical in restraining Bim activity (44), and Mcl-1 has been shown to be an important Bax/Bak antagonist, as the loss of Bax and Bak was able to rescue Mcl-1-deficient T cells, unlike the loss of Bim (43). Interestingly, Bax and Bak do not appear to have completely redundant functions, as Bim/Bak^{-/-} mice do not totally phenocopy Bim/Bax^{-/-} mice (310). This could potentially be due to differential specificity for or regulation of Bax/Bak among BH3-only proteins, expression differences in particular tissues, or the need for distinct inhibitory mechanisms for each due to their different conformations and localization within the cell (28).

Two competing models exist for how Bax/Bak activation occurs; in the indirect activation model, BH3-only proteins interact to sequester anti-apoptotic molecules from Bax and Bak, allowing their activation (46, 302). In the direct activation model, the 'activator' proapoptotic proteins Bim, tBid, and PUMA directly bind and activate Bax and Bak, which they are able to do by the action of 'sensitizer' BH3-only proteins such as Bad binding antiapoptotic Bcl-2 family members, freeing the activators from this sequestration (287, 311, 312). Nonetheless, it is likely that both mechanisms contribute differentially depending upon the context or both may contribute in the same cells in the same context (28, 313).

Maintaining T cell survival for therapeutic purpose

BH4 peptides

In addition to their ability to neutralize pro-apoptotic Bcl-2 family members, BH4-domain expressing Bcl-2 family members promote cell survival through mechanisms other than binding and inhibiting BH3-only pro-apoptotic molecules. For example, Bcl-2 and Bcl-xL bind to the inositol 1,4,5-trisphosphate receptor (IP3R) Ca^{2+} channel or voltage-dependent anion channel (VDAC) through their BH4 domain. These interactions further control cytoplasmic Ca^{2+} concentration and mitochondrial cytochrome c releasing, resulting in cell survival (Reviewed in (314)). Based on these BH4-dependent pro-survival mechanisms, BH4 peptides have been used as anti-apoptotic reagents (315).

Previous studies have shown that BH4 homologues can prevent human or mouse T cells from apoptotic cell death (316–318). However, the degree to which these cells were rescued remains unclear as cell death was not measured extensively. Further development of this approach may provide a clinical application in preventing T cell death. For example, BH4 homologues have been used to promote T cell survival in a pre-clinical mouse model of sepsis (319). On the other hand, inducing apoptosis through interrupting BH4 functions may be a potential treatment to eliminate unwanted T cells. For example, malignant T cells in lymphocytic leukemia diseases may be eliminated by this strategy (320).

IL-2

IL-2 is a common gamma chain cytokine that was discovered for its T cell stimulatory capacity, but later highlighted for its crucial role in the development, survival and function of Tregs (321). Mice deficient in IL-2 or IL-2 receptor develop systemic autoimmunity. IL-2 is crucial for the reciprocal balance between Th17 and Treg cells and also thought to impact the expansion and memory differentiation of CD8^{+} T cells (321).

As IL-2 has both immune activating and regulatory roles, the dosage of IL-2 is crucial for the type of response evoked, with lower doses promoting Treg expansion and higher doses evoking effector responses. IL-2 therapy was first explored in cancer trials, and high dose IL-2 showed promise in renal carcinoma and melanoma, though significant side effects and varying degrees of success were reported (322, 323). Subsequently, low dose IL-2 was found to increase Treg survival and prevented development of diabetes in a mouse model (324). Additionally, IL-2/IL-2mAb complexes, which lead to a slow and sustained IL-2 release *in vivo* led to an expansion of Tregs and were protective in mouse models of asthma (325) and EAE and islet allograft rejection (258). Consequently, low-dose IL-2 therapy has shown success in human clinical trials in HCV induced vasculitis (326), GVHD (327–329), T1D (330, 331), SLE (332) and Alopecia areata (333).

The mechanism of protection by IL-2 is likely complex, involving not only an enhanced survival and expansion of Tregs but also enhancing their suppressive function. Some studies investigated the impact on Treg survival upon IL-2 therapy. For example, Tregs after allogeneic HSC transplantation show decreased levels of Bcl-2 and increased susceptibility to Fas mediated apoptosis (327) and this was reversed by IL-2 therapy during chronic GVHD resulting in an improved outcome (328). Withdrawal of IL-2 is reported to cause

upregulation of the pro-apoptotic molecules Bim and Puma (334) and downregulation of Mcl-1. IL-2 therapy in combination with drugs like Rapamycin that cause death of T effectors might prove especially effective for selective targeting of Tregs (335). Combination therapies with IL-2 are being explored in transplantation and autoimmunity (336) and could prove vital in other areas like inflammation. Thus, given its ability to enhance Treg survival and function, the use of IL-2 could have applicability as a potential therapeutic from autoimmunity to transplant rejection.

IL-15

IL-15 is a member of the common γ -chain family of cytokines that is important for the development and survival of NK, NKT, CD8 effector and memory cells. One major mechanism by which IL-15 promotes survival is by maintaining expression of Bcl-2, the major Bim antagonist in effector CD8⁺ T cells (142). IL-15 was shown to promote CD8 $\alpha\alpha$ ⁺ IEL survival by both increasing Bcl-2 levels and dissociation of Bim from Bcl-2 (337). Recently, IL-15 was reported to improve the survival of CD8 memory T cells by regulating mitochondrial metabolism (338).

Given its role in promoting the survival and function of CD8⁺ T cells, IL-15 has been explored as an immunotherapeutic agent in certain forms of cancer or immunodeficiency states such as chronic HIV infection. For example, IL-15 co-delivered with HIV peptide vaccines also overcame CD4⁺ T cell deficiency by promoting longevity of antigen-specific CD8⁺ T cells via blocking TRAIL-mediated apoptosis through downregulation of Bax and upregulation of anti-apoptotic Bcl-XL (339). IL-15 along with vaccinia vectors expressing HIV gp160, supported robust CD8⁺ T cell-mediated long-term immunity in mice (340). A HIV vaccine adjuvanted with IL-15 significantly increased effector CD8 memory T cells and anti-HIV antibodies in monkeys compared to HIV vaccine alone controls (341). These findings suggest a clinical potential for IL-15, especially in individuals with CD4-deficiency (339). IL-15 therapy has also been investigated in other infectious diseases. In a *T. cruzi* infection model, the co-administration of a plasmid encoding IL-15 vaccination greatly improved long-term T cell survival, memory T cell expansion, and long-term protection against *T. cruzi* (342). In sepsis, IL-15 blocked the apoptosis of CD8⁺ T cells and increased the abundance of Bcl-2 while decreasing Bim and Puma (343). However, patients with sepsis with severe lymphopenia had downregulated Bcl-2 mRNA expression in peripheral blood mononuclear cells, despite increased plasma IL-15 concentrations (344). Thus, further studies are needed to understand the potential role of IL-15 in sepsis.

IL-15 also shows promise in clinical trials to treat patients with primarily non-hematological metastatic malignancy by boosting tumor surveillance. IL-15 is also an effective vaccine adjuvant in preclinical models of cancer and infectious diseases. IL-15 genetically expressed in a dendritic cell vaccine in a mouse breast cancer model enhanced antitumor responses and overcame defects in CD4⁺ T cell help (345). On the other hand, due to its effect on survival on B and T cells, IL-15 has a detrimental role in hematological malignancies and possibly autoimmunity and antagonism of IL-15 is being explored in this context (346). Thus, IL-15 shows promise as an adjuvant to enhance immune responses to tumors and infectious diseases.

IL-7

IL-7 promotes T cell development in the thymus and maintains survival of naive and memory T cells in the periphery (347). IL-7 has been shown to protect against death by upregulation of anti-apoptotic proteins Bcl-2 and Mcl-1 and by the inhibition of pro-apoptotic proteins Bax, Bad and Bim (348–352). In humans, IL-7 protects CD4⁺ effector/memory T cells from apoptosis induced upon the absence of stimulation and cytokines through the upregulation of Bcl-2, Bcl-xL and Mcl-1 (353). The robust capacity of IL-7 to enhance immune reconstitution of both CD4⁺ and CD8⁺ T cells makes it an attractive candidate in the clinical situations which would benefit from prevention of T cell death.

IL-7 therapy has been shown to augment T cell responses in cancer therapy, vaccination and infections. In tumor models, IL-7 therapy increased the survival of activated CD8⁺ T cells, the number of IL-17 secreting CD4⁺ T cell and CD8 memory responses with the greatest effect being on subdominant antigens (354, 355), suggesting a potential to increase immunity towards weak tumor antigens. In humans, IL-7 therapy increased circulating CD4⁺ and CD8⁺ T cell numbers and reduced Treg numbers in patients with metastatic melanoma (356, 357). The protective effect is attributable not only to enhanced survival and proliferation but also to enhanced function of T cells by IL-7's antagonizing action on inhibitory networks (358).

As IL-7 can promote T cell proliferation in lymphopenic conditions, it was proposed that exogenous IL-7 may be an important therapeutic following bone marrow transplantation, or in chronic HIV-1 infection. Exogenous IL-7 increased T cell cycling and survival which correlated with a sustained increase in Bcl-2 expression even after discontinuation of therapy in patients (359). IL-7 treatment of immunocompromised patients led to long term survival of CD4 and CD8⁺ T cells, including recent thymic emigrants as well as naive, memory, and central memory T cells demonstrating the usefulness of IL-7 during immune reconstitution after chemotherapy (360). IL-7 treatment of both SIV infected and uninfected non-human primates also augmented lymphocyte numbers considerably (361). IL-7 treatment during acute SIV infection was associated with sustained increase in the expression of Bcl-2 on both CD4⁺ and CD8⁺ T cells, expansion of CD8⁺ T-cells and development of antigen-specific responses (362). IL-7 therapy enhanced circulating CD4⁺ and CD8⁺ T cell numbers and homeostatic proliferation in HIV infected patients (363) and in patients undergoing ART, recombinant human IL-7 resulted in a dose-dependent, durable CD4⁺ T cell increase, associated with increased cell cycling and a transient increase in Bcl-2 expression (364). A variety of clinical trials using IL-7 are underway in patients with HIV or cancer reviewed here (347, 365).

IL-7 therapy also shows immense promise in the treatment of sepsis through prevention of T cell apoptosis. IL-7 enhanced T cell survival through upregulation of Bcl-2 and also mediated the cross talk between Th1 and Th17 lymphocytes improving the overall outcome (366). IL-7 therapy showed promise in a sepsis model by ameliorating CD4 and CD8⁺ T cell apoptosis likely through the upregulation of BCL-2 expression and decreased expression of Puma (367) and this protective effect was reproducible in humans (368). IL-7 also reduced apoptosis and improved survival in a 2-hit model of fungal sepsis which is clinically relevant in secondary hospital-acquired fungal infections suffering from sepsis (369).

IL-7 therapy also has potential to improve immune responses in aged populations as shown in aged Rhesus macaques, where treatment led to increase and long-term survival of central memory T cell (T_{CM}) (370). IL-7 therapy is further being explored in other contexts benefitting from T cell survival like hepatitis B and hepatitis C infections, in vaccines for aged populations and in adoptive T cell therapy for cancer. Taken together, there is immense promise for IL-7 to clinically augment immune reactivity without substantial side effects such as promoting inflammation. Conversely, IL-7's effect on T cell survival may also implicate it as a target for inhibition in autoimmune pathogenesis like RA and colitis (371).

Immunotherapy

Immunotherapy is emerging as a promising approach for cancer treatment, including the development of designed chemicals, biologics, or immune cells to enhance anti-cancer immune responses. One of the biologic approaches is using chimeric antigen receptor (CAR) T cells to initiate T cell-mediated antitumor immune responses (372, 373). The CAR T cells are generated from autologous or allogeneic T cells, transduced with viral vectors encoding the CAR. These CAR T cells are further expanded *ex vivo* and subsequently infused in patients.

One unsolved question for CAR T therapy is what determines the survival of CAR T cells in the patient body. In diverse treating conditions from different clinical trials, the persistence of CAR T cells is varied from few weeks to more than a year (373). The *in vivo* persistence may be manipulated by fine tuning T cell apoptotic mechanisms. For example, the intracellular tail of the CAR receptor proved crucial for the long-term persistence of the CAR transduced T cells. Reasoning that they would take advantage of co-stimulatory signaling, the intracellular tail from 4-1BB or CD28 (373) was used to provide an additional boost to the CAR T cells and likely does so by interfering with apoptosis. For instance, 4-1BB induces anti-apoptotic Bcl-2, Bcl-xL, and A1 (213, 374). Likewise, CD28 signals enhances the expression of Bcl-2 and Bcl-xL (244). In this vein, it has been known for years that Bcl-2 overexpression can promote tumor-specific T cell survival, and improve the efficiency of T cell immune therapy in a mouse model (375). Xu et al. reported that IL-7 and IL-15 treated CAR T cells have prolonged persistence in their mouse model, in line with higher survival rate during *ex vivo* expansion (376). In parallel, the oncolytic virus which additionally expresses IL-15 and RANTES prolongs CAR T persistence and increases the survival of tumor-bearing mice (377). Our and others studies have shown that IL-7 and IL-15 promote T cell survival through inducing Bcl-2 and/or repressing pro-apoptotic Bim, Puma, and Noxa. This information suggested a potential strategy optimize CAR T therapy.

Limiting T cell survival for therapeutic purposes

BH3 agonist

The increased structural understanding of Bcl-2 family member interactions has led to development of novel therapeutics, that inhibit the anti-apoptotic function of Bcl-2 family members, so called BH3 agonists (aka BH3 mimetics). These compounds mimic the structure of BH3 domains of pro-apoptotic Bcl-2 family members and sequester anti-apoptotic Bcl-2-like molecules, resulting in initiation of the mitochondrial apoptosis

pathway. Several BH3 agonists in clinical or preclinical studies for cancer therapy, including lymphoid malignancies, were recently reviewed in (378–380).

As discussed above, the Bcl-2 family is critical for T cell homeostasis, BH3 agonists may be applied to eliminate unwanted alloreactive or autoreactive T cells, in the case of transplantation or autoimmunity, respectively. For example, ABT-737, a BH3 agonist that specifically inhibits Bcl-2, Bcl-xL, and Bcl-w (381), has been tested in several autoimmune diseases models. ABT-737 was efficacious in driving T cell apoptosis and decreasing disease in murine inflammatory bowel disease (IBD) models (382), and a collagen-induced arthritis (CIA) mouse model (383, 384). Further, in an (NZB × NZW) F1 lupus model, ABT-737 treatment significantly decreases disease severity (384). Moreover, in an NOD mouse model, ABT-737 drove the death of autoreactive T cells, dendritic cells, and macrophages and prevented development of diabetes (385).

In addition, our group recently published a study showing that ABT-737 preferentially kills the accumulated B220⁺CD4⁺CD8⁺ T cells isolated from autoimmune lymphoproliferative syndrome (ALPS) patients, exhibiting less toxicity to other T cell subsets or T cells from healthy donors. We found that ABT-737 was effective in killing B220⁺CD4⁺CD8⁺ T cells linked to upregulation of Bcl-2 or Bcl-xL, which further indicates the potential of personalized treatment in eliminating harmful T cells (386). All these studies suggest that BH3 agonists have a strong potential in the therapy of autoimmune diseases. BH3 agonists are also good candidates to limit immune responses during organ transplantation. Recent studies showed that ABT-737 can target alloreactive T cells and prolong the graft survival in mouse skin transplant or pancreatic islet graft models (385, 387, 388). Unfortunately, ABT-737 drove substantial thrombocytopenia due to its ability to inhibit Bcl-xL, however, an improved revision, ABT-199, that retains binding to Bcl-2, but fails to bind to Bcl-xL, does not induce thrombocytopenia (389). Thus, in diverse auto immune and transplantation models, BH3 agonists have substantial therapeutic potential.

DNA damage response

T cell responses may also be limited for therapeutic purposes by utilizing drugs which damage DNA or manipulate DNA damage response (DDR) signaling. DNA may be damaged in a variety of ways, requiring an array of repair pathways, focusing on either DNA breaks (homology directed repair [HR], non-homologous end joining [NHEJ]), replication errors (mismatch repair [MMR]), or a variety of other chemical/structural lesions (base excision repair [BER], nucleotide excision repair [NER], the fanconi anemia pathway [FA], etc.). Defects in DNA repair pathways, mostly affecting NHEJ or HR (mutations of ATM, ATR, DNA-PK, Ligase IV, artemis, RAG) have been associated with primary immune deficiency (390). However, these disorders are largely due to defective T and B cell development (391) and DNA repair defects are not generally associated with abnormalities of peripheral T cell function (if they have developed normally). But notably, activated lymphocytes, are extraordinarily sensitive to DNA damaging chemotherapeutics or irradiation, perhaps because of their rapid division and genomic stress they endure when responding to pathogens (392).

Two major signaling pathways activated upon DNA breakage are the ATM-Chk2 checkpoint (which responds primarily to double stranded breaks (DSBs)) and the ATR-Chk1 checkpoint (activated principally in response to single stranded breaks (SSBs)) (393). Both of these pathways converge on cell cycle arrest and the activation of p53, the transcription factor which acts as the master regulator of the cell's response to genotoxic stress (394–397). Under normal conditions, p53 is kept at low amounts in the cell, sequestered in an inactive state by its regulator MDM2 (398–400). However, upon phosphorylation by ATM or ATR at Serine 15, p53 will dissociate from MDM2 and induce transcription of proteins responsible for mediating cell cycle arrest (i.e. p21) and apoptosis (such as Bax, PUMA, and Noxa) (276, 277, 401–404). The fate of the cell depends on the amount of active p53 accumulated in it, which in turn depends on the extent of DNA damage in the cell (405). The decision of whether to undergo cell cycle arrest and DNA repair or apoptosis depends on whether p53 reaches the so-called “apoptotic threshold,” the tipping point at which the cell will initiate apoptosis if it deems the damage to its genome too severe to repair (406). Multiple mechanisms of cell death may be triggered by p53 or other DNA damage signaling, including both the intrinsic and extrinsic pathways of apoptosis (407).

Consequently, DNA-damaging agents can be used to induce enough damage to the genomes of rapidly dividing cells so that they undergo apoptosis rather than simply arresting and repairing themselves; this is the rationale behind antineoplastic chemotherapeutics (24). Chemotherapy has also been utilized for decades to control the most serious forms of autoimmunity, allogeneic reaction, and immune dysregulation (408–410). However, the utility of this approach is severely limited by the toxicities of these agents. The fact that lymphocytes undergoing the explosive proliferation of an active immune response bear similarities to neoplasia suggest that manipulating DNA damage and repair may be an effective strategy to control aberrant immune responses, if toxicities can be modulated. Notably, a broad array of targeted agents effecting previously untargeted aspects of the DDR have been developed in recent years. We think some of these can be successfully repurposed for the treatment of many immune-mediated diseases. Notably, recent studies have shown that the topoisomerase II inhibitor etoposide is a remarkably potent agent for eliminating activated T cells in vivo and treating the immunoregulatory disease hemophagocytic lymphohistiocytosis (HLH) and the mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (411, 412). An interesting facet of these studies is the high degree of specificity exhibited by etoposide treatment in only depleting activated (i.e. pathogenic) T cells without significantly impacting naïve, memory, or regulatory T cells, which appear to be protected due to their much slower turnover rate and thus decreased sensitivity to DNA-damaging agents. This is likely in part due to the diminished expression of Bcl-2 in conjunction with increased levels of Bim in the activated T cell subset as compared to the other T cell populations. This imbalance places them at the brink of survival and the additional push given by etoposide is enough to drive them to apoptosis.

Though DNA damaging drugs such as etoposide may have notably selective effects on the immune system, they still suffer from off target toxicities, typically affecting the bone marrow or gut. However, recent data that we and our collaborators have generated has shown that not only do activated T cells have a pro-apoptotic balance of Bim and Bcl-2, but that they also show intrinsic activation of specific DDR pathways upon activation, a fact which

can be exploited therapeutically (McNally and Millen, et al., in press). By manipulating key proteins in particular pathways, it may be possible to therapeutically skew the fate of pathologic T cells towards apoptosis without having to induce damage more diffusely with genotoxic drugs. Small molecule inhibitors of the cell cycle checkpoint kinases Chk1/2 and Wee1 were developed for the treatment of cancers which lacked functional p53 and hence lacked an efficient G1/S cell cycle checkpoint. These cancers would be highly proliferative and highly dependent on the mid-S and G2/M cell cycle checkpoints to maintain genomic integrity. Since these checkpoints rely on Chk1/2 and Wee1, it was postulated that cancers would be especially sensitive to this targeted therapy. While these agents have proven to be effective and non-toxic in preclinical studies, clinical trials are ongoing (413). Because activated T cells proliferate at extraordinary rapid rates, we postulated that they would display a functional impairment of the G1/S checkpoint and also be especially sensitive to Chk1/Wee1 inhibition. Furthermore, because these cells have intact p53, they would also be sensitive to strategies which potentiate the action of p53, including inhibition of MDM2. We have tested these hypotheses and observe a clear sensitivity of activated T cells to cell cycle checkpoint inhibition and a combinatorial synergy with MDM2 inhibition in therapeutic contexts (McNally and Millen, et al., in press). This combination approach, which we term PPCA (**p**53 **p**otentiation with **c**heckpoint **a**brogation), effectively treats both EAE and murine HLH, and results in levels of pathogenic T cell ablation comparable to those achieved with etoposide, while avoiding substantial toxicity. This novel approach to modulating aberrant immune responses may represent a new therapeutic strategy in the treatment of a diverse array of immune-mediated diseases.

Summary

Over the last few decades, the molecular mechanisms underlying cell death processes are becoming well understood. Indeed, we have learned a great deal about individual Bcl-2 family members, their underlying biochemistry, and in many cases their tissue-specific roles in cellular homeostasis. Notably, this greater understanding has led to the development of “BH3 agonist” - a breakthrough in anti-cancer therapeutics. The fact that there is a substantial amount of specificity in the interactions between pro- and anti-apoptotic Bcl-2 family members bodes well for future therapeutic applications as off-target effects will be minimized. Further, as p53 is also a central mediator of apoptosis via the Bcl-2 family in response to DNA damage and the p53 pathway is often dysregulated in many types of cancers, the development of drugs manipulating p53 pathway function are being rapidly developed as anti-cancer therapies. Thus, a relatively underexplored area in the realm of immune modulation will be to exploit the oncologic pharmacopia in search of non-genotoxic strategies to push unwanted or deleterious lymphocytes over the apoptotic threshold and eliminate these clones from the repertoire.

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