Smyd1C Mediates CD8 T Cell Death via Regulation of Bcl2-Mediated Restriction of outer Mitochondrial Membrane Integrity

Hui Nie, Gary Rathbun, and Haley Tucker
Department of Molecular Biosciences and the Institute for Cellular and Molecular Biology, the University of Texas at Austin, Austin TX 78712, USA

Abstract

The SET and Mynd domain 1 (Smyd1) locus encodes three tissue-restricted isoforms. Two previously characterized isoforms, Smyd1A and Smyd1B, are heart and skeletal muscle-restricted histone methyl transferases. Here we report that a third, non-catalytic isoform, Smyd1C, is expressed predominantly in activated CD8 T cells. While Smyd1C-deficient CD8 T cells undergo activation-induced apoptosis, neither of two classical mechanisms activation-induced cell death nor activated cell autonomous death are utilized. Instead, Smyd1C accumulates within both mitochondria and the immunological synapse where it associates with Bcl-2, FK506-Binding Protein 8/38 (FKBP38) and Calcineurin. This complex maintains Bcl-2 phosphorylation, enhanced mitochondrial localization, and restricted apoptosis of activated CD8 T cells. We suggest that CD8 T cell death is governed, in part, by Smyd1C regulation of Bcl2-mediated restriction of outer mitochondrial membrane integrity.

Keywords

Cell death; Apoptosis; Phosphorylation; Smyd1

Introduction

Apoptosis is critical to the fate of T cells. In response to T cell receptor (TCR) stimulation, T cells exhibit an exponential rate of expansion followed by a rapid decline and a return to a basal pool. Failure to induce apoptosis leads to elevated T cell levels and, often, to inflammatory or autoimmune disorders [1]. Reciprocally, aberrant elimination of T cells can lead to immunodeficiency. Therefore, a fine balance between cell proliferation and programmed cell death is necessary to achieve proper T cell homeostasis. This balance is governed by two distinct apoptotic pathways: activation-induced cell death (AICD) and

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

*Corresponding author: Haley Tucker, Department of Molecular Biosciences, the University of Texas at Austin, 1 University Station AS000, Austin TX 78712, USA, Tel: (512) 475-7705; haleytucker@austin.utexas.edu.

Authorship Contributions
H.N. performed the experiments and wrote the original draft, G.R. consulted with H.N., and along with H.O.T. wrote the final draft of the paper.

Conflict of Interest Disclosure
The authors declare no conflict of interest.
activated T cell autonomous death (ACAD) [2]. While AICD is mediated through extrinsic pathways via the Fas death receptor or by signaling through TNF-α receptors, Bcl-2 family members play a central role in the removal of activated T cells by the ACAD mitochondrial intrinsic pathway [3–5]. During ACAD, a Bcl-2 family pro-apoptotic member, Bim, induces cell death [3]. Survival of the cell requires that anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-XL, block Bim by interacting with Bim at the mitochondrial membrane [5]. Thus, the relative levels of mitochondrial anti-apoptotic versus pro-apoptotic members of the Bcl-2 family critically influence the regulation of apoptosis [2,6,7].

Bcl-2 is anchored to the mitochondria through its association with the mitochondrial chaperone, FKBP38 [6,8]. FKBP38 contains a peptidylprolyl cis-trans isomerase (PPIase) domain through which it binds the immunosuppressive drug, FK506. While its effect on apoptosis of cultured cells is controversial, knockout (KO) studies demonstrated that, in vivo, FKBP38 is required for inhibiting cell death in the developing neural tube. Bcl-2 family proteins are regulated by reversible phosphorylation modifications that control their activity and conformation. PKCα is the major Bcl-2 kinase [2,9], whereas dephosphorylation of Bcl-2 by calcineurin (CaN) enhances its anti-apoptotic activity [10,11].

**Smyd1C** is a member of the Smyd family of proteins which are defined by the presence of a SET domain that is split into two segments by a MYND domain. The **Smyd1** gene encodes three distinct isoforms (Figure 1A) [12,13]. **Smyd1A** and **Smyd1B** are expressed exclusively in cardiac and skeletal muscle. They function in cultured cell lines as transcriptional repressors and in vitro as H3K4me3-specific histone methyl transferases (HMTases) [14,15]. **Smyd1A/B** null mice die early in embryogenesis from impaired cardiomyocyte differentiation and cardiac chamber morphogenesis [16]. **Smyd1C** transcripts were detected in T cell lines [12], but their function there was not further investigated. Relative to **Smyd1A** and **B**, **Smyd1C** is encoded by unique upstream promoter region and upstream exon 1, thereby severing the N-terminal half of the SET domain (Figure 1A and re-addressed in Results) [13].

In this study we evaluated the in vivo role of **Smyd1C** in T cells. We found that **Smyd1C** accumulates predominantly in the cytoplasm, mitochondria and immunological synapses of activated CD8 cells. **Smyd1C** conditional gene disruption led to impaired clonal expansion of CD8 T cell as a result of heightened levels of apoptosis. **Smyd1C** interacts with FKBP38, Bcl-2, and CaN, but has no HMTase activity toward them or toward conventional histone substrates. Instead, **Smyd1C** is required for dephosphorylation of Bcl-2 and for its efficient targeting to the mitochondrial membrane. Our data identify **Smyd1C** as a critical component of CD8 T cell death via a mechanism uniquely related to ACAD.

**Smyd1C** is devoid of histone methyl transferase (HMTase) activity and expressed exclusively in CD8+ DP and SP T cells

**Smyd1C** initiates transcription from a poorly consensus Kozak sequence (cccauga) located in the opposite translational orientation just 160bp centromeric to CD8β (Figure 1A). The resulting 31 residue exon 1 shares no significant similarity with any database entries (data not shown). **Smyd1C** exon 1 is spliced in frame to the second exon which is shared with its
two orthologues, *Smyd1A* and *B*). This eliminates the S segment of the SET domain (Figure 1A).

Severing the essential N-terminal half of the SET domain predicted that *Smyd1C* would lack HMTase activity. Indeed, that was the case (S-Figure 1A). However, as with its orthologues and paralogues, *Smyd1C* interacted with HDAC1 and displayed transcriptional repression on a synthetic substrate assayed by the Gal4-UAS system (Figures 1B and 1C). While this suggested that a transactivation domain might be retained, *Smyd1C* displayed no global gene expression alteration when over-expressed (data not shown). Thus, we conclude that *Smyd1C* unlikely plays a significant role in transcription.

It was previously reported [12] that *Smyd1C* expression was detected only in CD8+ cell lines and in thymus. Tissue expression survey confirmed that *Smyd1C* was expressed highly in thymus, modestly in spleen and strongly in CD8 T cell lines (Figures 1B and 1C). We further observed that *Smyd1C* transcripts in spleen were induced by Con A and dramatically induced when stimulated under conditions (detailed in Materials and Methods) of a secondary Mixed Lymphocyte Reaction (20 MLR) (Figure 1F, upper panel). *In vitro* 20 MLR mimics the allogeneic response of a recipient haplotype against donor MHC. To further examine the expression of *Smyd1C* in thymocyte subsets, mouse CD4 single-positive (SP), CD8SP, CD4CD8 double-positive (DP) and CD4 and CD8 double-negative (DN) thymocytes were isolated on respective magnetic beads. Levels of *Smyd1C*mRNA were analyzed by semi-quantitative RT-PCR. As predicted by its unique orientation downstream of the CD8 promoter, *Smyd1C* was expressed nearly exclusively in CD8 SP or DP T cells (Figure 1D).

**Smyd1C undergoes mitogen-stimulated activation in CD8 T cells**

We induced T cell activation by cross-linking the TCR/CD3 complex with immobilized anti-CD3 antibody along with co-stimulatory molecules (eg, anti-CD28) or with 12-phorbol 13-myristate acetate plus ionomycin (P+I). The latter approach bypasses cell-surface signaling by activating protein kinase C and increasing the intracellular Ca\(^{2+}\) concentration. We monitored *Smyd1C* transcript expression at 0, 24, 48 and 72 h post-stimulation by RT-PCR. As shown in Figure 1E, we observed upregulation of *Smyd1C*mRNA at 72 h following induction in splenocytes. This was accompanied by a gradual decline in *Smyd1C* transcripts in thymocytes at 24 h and a significant downregulation at 48 h.

It should be noted that all employed modes of stimulation were competent in upregulating *Smyd1C* expression in peripheral lymphocytes, suggesting that *Smyd1C* is an immediate downstream target of activated signaling. Up to 90% of cells in the thymus are DP immature thymocytes, and sustained TCR signaling leads to differentiation of those thymocytes to CD4SP T cells [17]. This is consistent with our observation that *Smyd1C* transcripts in thymocytes were reduced by prolonged TCR stimulation and subsequently expressed primarily in CD8 cells rather than CD4 cells (Figure 1D).

*Smyd1C* is expressed most highly in splenocytes following 6 days of mixed lymphocyte reaction (MLR) using C57BL/6 splenocytes as effectors and irradiated BALB/c splenocytes as targets (Figure 1F; details provided in Materials and Methods). Although the above
analyses detected elevated *Smyd1* transcripts, Western blotting allowed direct confirmation of *Smyd1C* protein expression, particularly following activation by P+I, ConA and MLR in splenic T cells (Figure 1G).

**Smyd1C partitions within mitochondria and peripheral supramolecular activation clusters of activated CD8 T cells**

Immunofluorescence microscopy initially indicated that *Smyd1C* is expressed exclusively within the cytoplasm of splenocytes stimulated by P+I (Figure 2A). Transfection of *Smyd1C* into nonlymphoid cell lines recapitulated this result, indicating that cytoplasmic localization is not T cell-restricted (Figure 2B). On closer inspection, we observed that, following P+I stimulation, *Smyd1C* localized in a punctate pattern, suggesting that at least a sub-fraction may reside in mitochondria. Mitochondrial localization was confirmed by staining with TMRE, a compound that loads specifically into polarized mitochondria (Figure 2C; readdressed below) [18].

Since allogeneic stimulation of peripheral T cells strongly induced *Smyd1C* expression (Figure 1D), we examined *Smyd1C* localization following 6 days of MLR, employing 4–6 wk old C57BL/6 splenocytes as effectors and irradiated BALB/c splenocytes as targets. Slides were fixed and initially stained for detection of *Smyd1C* and CD8. As shown in Figure 2D, a significant fraction of the cells formed stable T cell/APC conjugates. Similar results were obtained when CTL3, a CD8 T cell line, was employed as stimulator (S-Figure 2). Such conjugates are characteristic of supramolecular activation clusters (SMACs) typically observed at CTL–APC interfaces. Indeed, LFA-1, an integrin that marks the peripheral (pSMAC) cluster strongly colocalized with *Smyd1C* at the conjugate interface (Figure 2F).

Previous studies showed that during T cell activation, mitochondria mobilize towards the vicinity of the immune synapse [19–21]. Re-inspection of the images of (Figure 2C) indicated that a large fraction of *Smyd1C* and mitochondria (indicated by TMRE fluorescence) colocalized at cell–cell contacts. Taken together, our data suggest that *Smyd1C* is a critical component of mitochondrial-pSMAC alignment and argue for potential non-nuclear signaling function for *Smyd1C* during the early stages of T cell activation.

**T cell-specific conditional deletion of Smyd1C**—Germline deletion of *Smyd1* results in embryonic lethality at E9.5, owing to cardiac defects [16]. Transcripts of the two major isoforms, *Smyd1A* and *Smyd1B*, are undetectable in thymus and spleen (S-Figure 3D) [13,16]. This allowed us to analyze the sole function of *Smyd1C* function within the T cell lineage. We employed a conditional cre/lox approach (detailed in Materials and Methods and in S-Figure 3) in which a floxed *Smyd1C* allele created in C57BL.6 was crossed into B6.Cg-Tg Lck-cre 548 Jxm/J driver mice. This system was previously shown to delete CD4 SP and CD4+CD8+ DP cells efficiently, and CD8+ SP cells robustly [22,23].

To confirm *Lck-Cre* specificity in our context, total RNA and genomic DNA from unfractionated thymocytes and other tissues, were examined by semi-quantitative RT-PCR or by end point-PCR using primers that distinguish WT, floxed, Cre, and deleted alleles. Figure 3A and S-3D show independent and representative examples of *Lck-Cre*-mediated deletion...
in the thymus and spleen. We estimated that Smyd1C expression in thymus and spleen were reduced ~80%, although WT expression levels in spleen are quite low. Adult Smyd1C conditional knockout (CKO) mice were indistinguishable from wild type (WT) littermates or from littermates bearing Lck, Cre or floxed Smyd1C alleles alone in viability and overall phenotype (data not shown).

**Resting T cell levels and developmental markers are unaffected by Smyd1C loss**

Given the highly temporal and T cell-restricted expression pattern of Smyd1C, our initial investigation focused on developing thymocytes. In the thymus, immature T cells develop by progressing through distinct stages that are characterized by the expression of different sets of regulatory cell-surface proteins. Mature CD4 and CD8 SP subsets home to peripheral lymphoid tissues [24]. Total thymocytes from Smyd1C WT and CKO mice were compared for expression of CD4CD8 DP and SP subsets as well as for T cell development/activation markers, including CD25, CD44, CD3, CD5, CD69 and CD24. FACS profiles indicated that neither T cell subsets nor development markers were significantly altered in splenocytes or in thymocytes of CKO mice relative to WT controls (Figures 3B and 3C; S-). This indicated that Smyd1C is not required for T cell development.

**Stimulated Smyd1C-deficient CD8 T cells are activation deficient**

Based on its restricted expression in CD8 T cells and its ability to undergo up-regulation in response to stimulatory signals, we reasoned that Smyd1C may function preferentially in activated T cells [12]. Total splenocytes, excluding erythrocytes, derived from Smyd1C WT and CKO mice were incubated with P+I or anti-CD3+anti-CD28 to activate T cells. Cells were harvested on days 3 and 5, post-stimulation, and stained with antibodies against CD4 and CD8 as well as against T cell activation markers (CD25, CD44, and CD69). We observed that both percentages and absolute numbers of CD8+ CKO splenocytes and thymocytes were markedly reduced (Figure 4A and S-Figure 5). We also observed downregulation of CD69 and CD25 on gated CKO CD8 T cells (Figure 4B). CD69, a C-type lectin of unknown ligand specificity, is a very early activation marker upregulated on all leukocytes following Ag encounter [25,26]. CD25, a component of the trimeric interleukin 2 receptor (IL-2R), is transiently expressed by CD4+ and CD8+ T cells following TCR activation [27]. CD25 appears does not participate directly in ligand binding, but, instead, functions to increase the ligand affinity of IL-2R 10–100-fold [27]. Smyd1C-mediated reduction of both CD69 and CD25 is mechanistically linked in that activation of CD69 has been shown to induce CD25 upregulation on CD8+ T cells [28]. However, Smyd1C loss did not impair the ability of stimulated splenic T cells to synthesize IL-2 and INF-γ as determined by intracellular staining and RT-PCR (S-Figure 6 and data not shown).

Thus, we reasoned that an underlying contributor to the above phenotype might be failure of activated Smyd1C-deficient CD8 splenocytes to proliferate. To evaluate, magnetic bead-isolated CD8 splenocytes from Smyd1C CKO and WT mice were stimulated with anti-CD3+anti-CD28 and subjected to 12hr intracellular incorporation of BrdU into newly synthesized DNA. FACS analysis detected no significant deficiency in Smyd1C CKO incorporation into CD8 or CD4 T cells (Figure 5A). However, in an alternative approach, in which we measured cell proliferation by 3H-thymidine incorporation via several conditions,
detected a modest reduction (statistically, a trend towards significance; p ≤0.07) in proliferation of P+I stimulated CKO CD8 splenocytes (Figure 5B). Different outcomes using these two types of proliferation analyses have been previously observed [29]. Collectively, we conclude that activated Smyd1C-deficient CD8 T cells proliferate at near normal levels.

**Smyd1C-deficient CD8 T cells are impaired in killing and in target cell elimination**

To assess whether Smyd1C deficiency perturbs target-cell elimination mediated by cytotoxic T lymphocytes (CTLs), mice were infected with the murine Lymphocytic Choriomeningitis Virus (LCMV; detailed in Materials and Methods). LCMV elicits a vigorous CTL response against a defined array of MHC class I–restricted viral epitopes [30]. Target EL4 cells were labeled with a fluorescent probe and pulsed with nucleoprotein epitope NP396–404. These were then co-incubated with fresh splenocytes obtained from CKO and control mice 8 days following LCMV infection in the presence of a fluorogenic caspase substrate to allow detection of substrate cleavage by FACS. As shown in Figure 5C, the number of target cells undergoing caspase cleavage in Smyd1C CKO mice was moderately but significantly reduced relative to WT.

CTL-mediated killing is mechanistically related to granular release by CTL during recognition. Consistent with the above reduction in caspase cleavage, the activity of BLT esterase, a substrate used as a measure of granzyme release, was significantly reduced in CKO mice relative to WT controls (Figure 5D). However, the mRNA levels of two main granular components, Granzyme B and perforin showed no significant reduction (S-Figure 6).

These data indicated that Smyd1 CKO mice suffer a principle defect in CD8-mediated killing, albeit conventional factors that mediate granular release are unscathed.

**Smyd1C-deficient CD8 T cells undergo activation-induced apoptosis—**

Activation of Caspase 3, the “executioner” of cell death, through proteolytic cleavage of its inactive zymogen into activated p17, plays a central role in apoptosis. Therefore, we monitored cleavage of caspase-3 in response to TCR signaling. Using isolated CD8 T cell whole cell lysates stimulated for 3 days with P+I, immunoblotting with anti-Caspase 3 mAb revealed that cleavage of CKO CD8 T cells was strongly reduced (Figure 5F).

These data suggested that Smyd1C inhibits activated CD8 T cells from initiating apoptosis and explains, at least in a major part, why CD8 cell numbers were severely reduced by Smyd1C loss (Figure 4A) yet proliferative indices were, at best, only modestly impaired (Figures 5A and 5B).

**Smyd1C employs neither classical AICD nor ACAD to restrict CD8 T cell responses**

As the immune response wanes, activated lymphocytes are removed in two ways: Those re-stimulated near the end of the immune response die by activation-induced cell death (AICD), whereas those activated, but not re-stimulated, die by activated cell autonomous death (ACAD) [1–4]. Several features noted above suggest that Smyd1C does not promote AICD. These include failure to activate IL-2, IFNγ and CD96/FAS—quintessential markers of AICD (S-Figure 6) [1–4]. Further, CTLA-4, which elicits a potent block of FasL...
expression and AICD [30] was unaffected by Smyd1C CKO (S-Figure 6). These results were consistent with previous observations that AICD does not require TCR re-stimulation and is independent of death receptor engagement [2].

ACAD is typically regulated by the NF-κb family as well as by the intrinsic cell death pathway involving members of the Bcl-2 family [1,2,4,31,32]. However, mRNA levels of both pro- (e.g., Bim, Bid, Bcl-XL) and anti- (e.g., Bcl2, BclXL and Mcl-1) apoptotic Bcl-2 family members as well as their effectors (caspases 3 and 8) were unaffected by Smyd1C CKO (S-Figure 6).

These results argue against Smyd1C as a regulator of either AICD-or ACAD-dependent CD8 T cell responses. Its action is independent of IL-2, IFNg and death receptor signaling, and the expression of pro or anti-apoptotic Bcl-2 family members is not disrupted.

**Association of Smyd1C with the immunophilin FKBP38 enhances mitochondrial localization of Bcl-2**

To search for an alternative mechanism by which Smyd1C protects against CD8 death, we performed yeast two-hybrid screening using Smyd1 as a bait to screen a mouse T cell cDNA library (Materials and Methods). Among the positive candidates, we identified FK506-Binding Protein (FKBP38; S-Figure 7. FKBP38), an immunosuppressant of FK-506 Binding Protein, serves an important function in mitochondria-mediated apoptosis by regulating anti-apoptotic Bcl-2 [5,33,34] This prompted us to examine if the expression of FKBP38 and Bcl-2 were affected by loss of Smyd1C. This was not the case, as analysis of P+I activated splenocytes revealed similar levels of FKBP38 and Bcl-2 protein and transcripts in Smyd1C CKO and WT mice (Figure 6 and data not shown).

Binding of FKBP38 to Bcl-2 has been shown to induce mitochondrial localization of their complex [5,33]. Thus, we reasoned that Smyd1C might regulate apoptosis by altering the intracellular localization of FKBP38, Bcl-2 or both. Under physiological conditions, both FKBP38 and Bcl-2 localize primarily within mitochondria, although smaller quantities are found in ER, Golgi and the nuclear envelope. Double IF staining of P+I activated splenic T cells revealed that the mitochondrial localization of Bcl-2 was more prominent in Smyd1C WT than in CKO cells (Figure 6A). We observed no significant difference for FKBP38 localization (Figure 6B). To confirm these results, we isolated [28]; mitochondria from activated peripheral T cells. As shown in Figure 6C, mitochondrial Bcl-2 was significantly reduced, while cytoplasmic Bcl-2 was significantly increased in Smyd1C CKO cells. Localization of FKBP38 again showed only modest alteration (Figure 6C). These observations suggested that Smyd1C promotes mitochondrial localization of Bcl-2.

**Smyd1C associates with Bcl-2 and FKBP38**

Next we examined whether Bcl-2 associates with Smyd1C. Following co-transfection of Smyd1C and Bcl-2 into NIH3T3 cells, both transfected and endogenous (red circled) Bcl-2 were detected in anti-Smyd1 immunoprecipitates (IPs; Figure 6D, left panels). Reciprocally, anti-Smyd1C IPs also contained both transfected and endogenous (red circled) FKBP38 (Figure 6D, right panels). Finally, following triple-transfection of Smyd1C, FKBP38, and Bcl-2 into NIH3T3 cells, each were IP’d by Smyd1C (Figure 6E). The Smyd1C doublets
observed in Figures 6D and 6E likely represent phosphorylated and unphosphorylated forms (readdressed below).

These results raised the possibility that Smyd1, Bcl-2, and FKBP38 reside in the same complex. They further suggest that Smyd1C regulates Bcl-2 localization by associating directly, or indirectly with it and Bcl-2. As Bcl-2 anti-apoptosis activity is dependent upon its mitochondrial localization, such association would provide a mechanism by which Smyd1C regulates apoptosis in activated CD8 cells (readdressed below).

**Smyd1C enhances phosphorylation of Bcl-2 while associating with Calmodulin**

Proteins are often regulated by post-translational modifications that control their activity and conformation. In most instances, phosphorylation of Bcl-2 has been associated with its inactivation and restriction of its localization predominantly to the ER [30–32]. This led us to hypothesize that reduced mitochondrial occupancy of Bcl-2 in Smyd1C-deficient mice may result from alteration in its phosphorylation state. To address this, P+I activated peripheral T cells were labeled with $[^{32}P]$-orthophosphoric acid and then IP’d using a polyclonal Bcl-2 antibody. The IPs were split into two fractions, and each was fractionated by SDS/PAGE. Half was exposed to autoradiography (16hr at −80°C), while the other half was subjected to immunoblotting with a Bcl-2 mAb. As shown in Figure 7A, the level of phosphorylated Bcl-2 was significantly increased in Smyd1C CKO T cells.

Association of Bcl-2 with the calcium-calmodulin-dependent protein phosphatase Calcineurin/PP2B phosphatase (CaN) was shown to prevent TCR-mediated apoptosis by blocking intracellular calcium signaling [35,36]. CaN was further shown to bind directly to both Bcl-2 and FKBP38 [33,37–39]. As shown in Figure 7B, CaN also immunoprecipitated specifically with Smyd1C, but not Smyd1A or B, in CD8+ splenocytes. We find it noteworthy in this regard that a measurable loss in CaN-mediated phosphatase activity was observed in lysates prepared from stimulated CKO CD8 splenocytes (Figure 7C).

**Hypothetical mechanism by which activated T cell fate is regulated by Smyd1C**

Our data indicate that reduction of Smyd1C triggers an earlier onset of activated T cell death and results in an abbreviated immune response. In Figure 7D, we illustrate an unconventional ACAD model for the underlying mechanism. We suggest that Smyd1C acts as a scaffold for Bcl-2, FKBP38 and CaN. This hypothetical complex insures Bcl-2 phosphorylation-based mitochondrial association and anti-apoptosis of activated CD8 T cells. In turn, loss/down-regulation of Smyd1C expression leads to dissociation of the complex, CaN-mediated dephosphorylation of Bcl-2 and consequently, activation-induced apoptotic cell death. This model is readdressed in the Discussion.

**Discussion**

TCR stimulation of primary T cells generates a pool of active effector cells through a process of activation and expansion, followed by cell death [37,38,40]. It is important to regulate the onset of cell death to maintain a balance between the effector phase and subsequent cell death. An abbreviated effector phase could result in an inadequate immune response, whereas a prolonged effector phase could lead to the accumulation of overly...
activated cells. The mechanism by which this balance is controlled is not well understood. While it is known that Fas signaling contributes to the death of some activated T cells, it certainly does not control the death of all, or even most, as indicated by several reports that have shown that activated T cells display little variation in their rate of death in the absence of Fas signaling [3,41,42]. Experiments by others strongly suggest that many activated T cells die through an intrinsic cell death pathway (Activated Cell Autonomous Death; ACAD). ACAD often is executed via Bcl-2 family members and mitochondrial release of cytochrome C [41–45]. The death of the majority of activated T cells responding to foreign antigen in vivo can be prevented by over-expression of Bcl-2 [42,45]. Bcl-2 inhibits ACAD by associating with Bim at the mitochondrial membrane to block its apoptotic function and/or to alter permeabilization of the outer mitochondrial membrane [33,46]. The molecular mechanism(s) that control this intrinsic cell death pathway are unclear.

In contrast to its muscle-specific, enzymatically active isoforms, Smyd1C is exclusively expressed in CD8 SP thymocytes, splenocytes and some CTL cell lines (Figures 1C–1G) [12,13]. Expression of Smyd1C is robustly induced by TCR stimulation (Figures 1D–1G). While there are no obvious phenotypic alterations found in Smyd1C-deficient resting T cells, their CD8 cells respond to TCR stimulation with a high rate of cell death (Figures 4A, 5C and 5E). Smyd1C lacks the essential N-SET domain and thus, demonstrates no HMTase activity (S-Figure 1). Thus, we searched for a non-enzymatic mechanism. We found that Smyd1C acts via a new twist on the classical Activated Cell Autonomous Death (ACAD).

Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum (ER), and nuclear envelope [6,47]. The mitochondrial localization of Bcl-2 is essential for its anti-apoptotic function [6,8]. Evidence has shown that FKBP38 regulates apoptosis through interacting with Bcl-2 and anchoring it on the mitochondrial membrane [48,49]. Our results show that Smyd1C interacts with endogenous FKBP38 and Bcl-2 in co-immunoprecipitation experiments from cultured mammalian cells (Figures 6D and 6E). We further demonstrate that the mitochondrial localization of FKBP28 is normal, whereas that of Bcl-2 is significantly reduced in activated CD8 T cells of Smyd1C CKO mice (Figures 6A–6C). This suggested that Smyd1C inhibits apoptosis by targeting Bcl-2 to the mitochondria.

In vivo, Bcl-2 exists in varying states of phosphorylation and these states influence its subcellular location, its binding to pro-apoptotic family members, and ultimately, its anti-apoptotic activity [50–52]. Phosphorylated Bcl-2 is known to be predominantly localized within the ER where it is prevented from binding to the BH3-only pro-apoptotic protein Bim [50,53]. Bcl-2 is also known to prevent TCR-mediated apoptosis by blocking intracellular calcium signaling [54]. This results from its association with Calcineurin (CaN), a calcium-calmodulin-dependent protein phosphatase that was found to bind directly to both Bcl-2 and FKBP38 [6,37–39]. FKBP38 is an inherent inhibitor of CaN and was found to promote protein dephosphorylation [27]. In addition, activated CaN results in neutralization of the anti-apoptotic action of Bcl-2 [6,37–39]. We found that CaN interacts with Smyd1C, and its phosphatase activity is impaired following Smyd1C CKO or Smyd1B knockdown in C2C12 myocytes (Figures 7B and 7C; data not shown). As anticipated from CaN enzymatic loss,
phosphorylated Bcl-2 levels were significantly elevated in activated CD8 splenocytes (Figure 7A).

Taken together, these findings suggest that Bcl-2 may be regulated by Smyd1C through its association with FKBP38 and calcineurin. Perhaps these three proteins function in a ternary complex, although such stoichiometry remains to be formally established.

We propose in Figure 7D the following model: In resting T cells, which express modest levels of Smyd1C, the balance between survival and death of T cells is maintained by a complex set of ACAD and AICD mechanisms. However, in stimulated CD8 T cells, an increase of intracellular calcium ions activates CaN which, in turn, forms a complex with Bcl-2 (or potentially a Bcl-2-Smyd1C complex). As the level of Smyd1C is gradually up-regulated upon stimulation, it may exert a dominant negative effect by interacting with, and thereby sequestering, FKBP38. This results in de-repression of CaN by FKBP38. De-repressed CaN then can induce dephosphorylation of Bcl-2, potentially enhanced binding to Bim, and mitochondrial localization of Bcl-2. Thus, apoptosis is prevented, and CD8 T cells are maintained in an adequate effector phase.

FKBP38 binds to mTOR and inhibits its anti-apoptosis activity [55,56]. Thus, anti-apoptosis achieved via Smyd1C through a potential FKBP38-mTOR pathway cannot be excluded and should be investigated in the future. Further studies are required to determine how Smyd1C controls the phosphorylation state of Bcl-2 and to better define the role of Smyd1C at the immunological synapse. Finally, we suggest that extension of these studies may lead to better understanding of how activated T cells escape apoptosis in autoimmune disorders or in T cell lymphomas [1,47].

Methods

Mice protocols/approvals and description of T cell lines [24,34,57–68] Bai et al. [17] and Takayama et al., [18] are provided in Appendix 1. Details of our conditional T cell knockout (CKO) of Smyd1C employing B6.Cg-TgLck-cre548Jxm/J driver mice [22,23] and genotyping approaches [17] for screening are detailed in Appendix 1. Standard implementation of suspension cultures, mixed lymphocyte reaction (MLR) [12,34] and flow cytometry [12,63] were previously described and detailed in Appendix I. Immunoprecipitation and western blotting details were described previously [14,15]. Mitochondria were isolated and stained by the method of [28]. CTL assays were as described previously [14,15,68]. BLT-esterase activity was measured in supernatants as described by Takayama et al. [66]. In vivo metabolic labeling and IP were previously described [14,15]. The Matchmaker Gold Yeast Two-Hybrid 63system was used to screen for Smyd1C binding partners. Calcineurin Phosphatase activity was determined by a colorimetric kit (Abcam#13946).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

We thank June V. Harriss for expert assistance in the generation of Smyd1 conditional knockout mice, and Chhaya Das and Maya Ghosh for help in wet lab and cell culture experiments, and Dr. Li Zhou for helpful discussions. NIH Grant R01CA31534; Cancer Prevention Research Institute of Texas (CPRIT) Grants RP120348 and RP120459; and the Marie Betzner Morrow Centennial Endowment (to H.O.T.) provided support for this work. We thank the late Paul Gottlieb for initiating this study.

References

1. Arnold R, Brenner D, Becker M, Frey CR, Krammer PH. How T lymphocytes switch between life and death. Eur J Immunol. 2006; 36:1654–1658. [PubMed: 16791883]
2. Brenner D, Krammer PH, Arnold R. Concepts of activated T cell death. Crit Rev Oncol Hematol. 2008; 66:52–64. [PubMed: 18289867]
3. Hildeman DA, Zhu Y, Mitchell TC, Kappler J, Marrack P. Molecular mechanisms of activated T cell death in vivo. Curr Opin Immunol. 2002; 14:354–359. [PubMed: 11973134]
4. Marrack P, Kappler J. Control of T cell viability. Annu Rev Immunol. 2004; 22:765–787. [PubMed: 15032596]
5. Sivakumar D, Sivaraman T. A Review on Structures and Functions of Bcl-2 Family Proteins from Homo sapiens. Protein Pept Lett. 2016; 23:932–941. [PubMed: 27449944]
6. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat Cell Biol. 2003; 5:28–37. [PubMed: 12510191]
7. Klumpp S, Kriegstein J. Serine/threonine protein phosphatases in apoptosis. Curr Opin Pharmacol. 2002; 2:458–462. [PubMed: 12127881]
8. Wang HQ, Nakaya Y, Du Z, Yamane T, Shirane M, et al. Interaction of presenilins with FKBP38 promotes apoptosis by reducing mitochondrial Bcl-2. Hum Mol Genet. 2005; 14:1889–1902. [PubMed: 15905180]
9. Ruvolo PP, Deng X, Carr BK, May WS. A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. J Biol Chem. 1998; 273:25436–25442. [PubMed: 9738012]
10. Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol. 1998; 18:8469–8478. [PubMed: 10567572]
11. Chang BS, Minn AJ, Muchmore SW, Fesik SW, Thompson CB. Identification of a novel regulatory domain in Bcl-X(L) and Bcl-2. EMBO J. 1997; 16:968–977. [PubMed: 9118958]
12. Hwang I, Gottlieb PD. Bop: a new T-cell-restricted gene located upstream of and opposite to mouse CD8b. Immunogenetics. 1995; 25:353–361. [PubMed: 7590968]
13. Hwang I, Gottlieb PD. The Bop gene adjacent to the mouse CD8b gene encodes distinct zinc-finger proteins expressed in CTLs and in muscle. J Immunol. 1997; 158:1165–1174. [PubMed: 9013956]
14. Tan X, Rottman J, Li H, De Deyne P, Du SJ. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. Proc Natl Acad Sci USA. 2006; 103:2713–2718. [PubMed: 16477022]
15. Sirinupong N, Brunzelle J, Ye J, Pirzada A, Nico L, Yang Z. Crystal structure of cardiac-specific histone methyltransferase SmyD1 reveals unusual active site architecture. J Biol Chem. 2010; 285:40635–40644. [PubMed: 20943667]
16. Gottlieb PD, Pierce SA, Sims RJ, Yamagishi H, Weihe EK, et al. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. Nat Genet. 2002; 31:25–32. [PubMed: 11923873]
17. Singer A. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. Curr Opin Immunol. 2002; 14:207–215. [PubMed: 11869894]
18. Solaini G, Sgarbi G, Lenaz G, Baracca A. Evaluating mitochondrial membrane potential in cells. Biosci Rep. 2007; 27:11–21. [PubMed: 17497220]
19. Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, et al. T cell activation requires mitochondrial translocation to the immunological synapse. Proc Natl Acad Sci USA. 2007; 104:14418–14423. [PubMed: 17726106]

20. Schwindling C, Quintana A, Krause E, Hoth M. Mitochondria positioning controls local calcium influx in T cells. The mitochondrial fission factor dynamin-related protein 1 modulates T-cell receptor signalling at the immunological synapse. J Immunol. 2010; 184:184–190. [PubMed: 19949095]

21. Baixauli F, Martín-Cófreses NB, Morlino G, Carrasco YR, Calabia-Linares C, et al. The mitochondrial fission factor dynamin-related protein 1 modulates T-cell receptor signalling at the immune synapse. EMBO J. 2011; 30:1238–1250. [PubMed: 21326213]

22. Orban PC, Chui D, Marth JD. Tissue- and site-specific DNA recombination in transgenic mice. Proc Natl Acad Sci U S A. 1992; 89:6861–6865. [PubMed: 1495975]

23. Shi J, Petrie HT. Activation kinetics and off-target effects of thymus-initiated cre transgenes. PLoS One. 2012; 7:e46590. [PubMed: 23049709]

24. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. J Immunol. 1982; 129:2293–2300. [PubMed: 6181166]

25. da Rocha Dias S, Rudd CE. CTLA-4 blockade of antigen-induced cell death. Blood. 2001; 97:1134–1137. [PubMed: 11159548]

26. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, et al. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 2002; 415:922–926. [PubMed: 11859372]

27. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. 2001; 15:1481–1486. [PubMed: 11410528]

28. Chappell JB, Hansford RG. Preparation of mitochondria from animal tissues and yeasts in Subcellular Components-Preparation and Fractionation London. Butterworths. 1972:77–91.

29. Pagliarini DJ, Clavo SE, Chang B, Sheth SA, Vafail SB, et al. A mitochondrial protein compendium elucidates complex I disease biology. Cell. 2008; 134:112–123. [PubMed: 18614015]

30. Ito T, Deng X, Carr B, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem. 1997; 272:11671–11673. [PubMed: 9115213]

31. Pommimaniit PB, Chen B, OltvaiZN. Interleukin-3 induces the phosphorylation of a distinct fraction of bcl-2. J Biol Chem. 1999; 274:1033–1039. [PubMed: 9873048]

32. Bassik MC, Scorrano L, Oakes SA, Pozzan T, Korsmeyer SJ. Phosphorylation of BCL-2 regulates ER Ca2+ homeostasis and apoptosis. EMBO J. 2004; 23:1207–1216. [PubMed: 15010700]

33. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol. 2003; 195:158–167. [PubMed: 12652643]

34. Richie ER, McEntire BB, Coghlan L, Poenie M. Murine T-lymphomas corresponding to the immature CD4-8+ thymocyte subset. Dev Immunol. 1991; 1:255–264. [PubMed: 1840416]

35. Zhong F, Davis MC, McColl KS, Distelhorst CW. Bcl-2 differentially regulates Ca2+ signals according to the strength of T cell receptor activation. J Cell Biol. 2006; 172:127–137. [PubMed: 16391001]

36. Erin N, Bronson SK, Billingsley ML. Calcium-dependent interaction of calcineurin with Bcl-2 in neuronal tissue. Neuroscience. 2003; 117:541–555. [PubMed: 12617961]

37. Van Parijs L, Biuckians A, Abbas AK, Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes. J Immunol. 1998; 160:2065–2071. [PubMed: 9498742]

38. Van Parijs L, Peterson DA, Abbas AK. The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. Immunity. 1998; 8:265–274. [PubMed: 9492007]

39. Hildeman DA, Mitchell T, Kappler J, Marrack P. T cell apoptosis and reactive oxygen species. J Clin Invest. 2003; 111:575–581. [PubMed: 12618509]

40. Strasser A, Harris AW, Huang DC, Krammer PH, Cory S. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. 1995; 14:6136–6147. [PubMed: 8557033]

41. Kroemer G. Mitochondrial implication in apoptosis. Towards an endosymbiont hypothesis of apoptosis evolution. Cell Death Differ. 1997; 4:443–456. [PubMed: 16465265]
42. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, et al. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. 1993; 53:4701–4714. [PubMed: 8402648]

43. Edlich F, Weiwad M, Erdmann F, Fanghänel J, Jarczowski F, et al. Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. EMBO J. 2005; 24:2688–2699. [PubMed: 15990872]

44. Shirane M, Ogawa M, Motoyama J, Nakayama KI. Regulation of apoptosis and neurite extension by FKBP38 is required for neural tube formation in the mouse. Genes Cells. 2008; 13:635–651. [PubMed: 18459960]

45. Bai X, Ma D, Liu A, Shen X, Wang QJ, et al. Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science. 2007; 318:977–980. [PubMed: 17991864]

46. Murali AK, Mehrotra S. Apoptosis - an Ubiquitous T cell Immunomodulator. J Clin Cell Immunol. 2011; S3:2. [PubMed: 23504027]

47. Rashedi I, Panigrahi S, Ezzati P, Ghavami S, Los M. Autoimmunity and apoptosis--therapeutic implications. Curr Med Chem. 2007; 14:3139–3151. [PubMed: 18220747]

48. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, et al. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity. 2002; 16:759–767. [PubMed: 12121658]

49. Loyola A, LeRoy G, Wang YH, Reinberg D. Reconstitution of recombinant chromatin establishes a requirement for histone-tail modifications during chromatin assembly and transcription. Genes Dev. 2001; 15:2837–2851. [PubMed: 11691835]

50. Shitashige M, Toi M, Yano T, Shibata M, Matsuo Y, Shibasaki F. Dissociation of Bax from a Bcl-2/Bax heterodimer triggered by phosphorylation of serine 70 of Bcl-2. J Biochem (Tokyo). 2001; 130:741–748. [PubMed: 11726273]

51. Shibasaki F, Kondo E, Akagi T, McKeon F. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature. 1997; 386:728–731. [PubMed: 9109491]

52. Lissy NA, Van Dyk LF, Becker-Hapak M, Vocero-Akbani A, Mendler JH, et al. TCR antigen-induced cell death occurs from a late G1 phase cell cycle point. Immunity. 1998; 8:57–65. [PubMed: 9462511]

53. Fournel S, Genestier L, Robinet E, Flacher M, Revillard JP. Human T cells require IL-2 but not G1/S transition to acquire susceptibility to Fas-mediated apoptosis. J Immunol. 1996; 157:4309–4315. [PubMed: 8906804]

54. Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, et al. Reactive oxygen species regulate activation-induced T cell apoptosis. Immunity. 1999; 10:735–744. [PubMed: 10403648]

55. Zou H, Lai Y, Zhao X, Yan G, Ma D, et al. Regulation of mammalian target of rapamycin complex 1 by Bcl-2 and Bcl-XL proteins. J Biol Chem. 2013; 288:28824–28830. [PubMed: 23960074]

56. Yoon MS, Sun Y, Arauz E, Jiang Y, Chen J. Phosphatidic acid activates mammalian target of rapamycin complex 1 (mTORC1) kinase by displacing FK506 binding protein 38 (FKBP38) and exerting an allosteric effect. J Biol Chem. 2011; 286:29568–29574. [PubMed: 21737445]

57. Clarke SRM, Barnden M, Kurts C, Carbone FC, Miller JF, et al. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. Immunol Cell Biol. 2000; 78:110–117. [PubMed: 10762410]

58. Guimezanes A, Montero-Julian F, Schmitt-Verhulst AM. Structural and kinetic basis for low affinity cross-reactivity in T cell allorecognition. Eur J Immunol. 2003; 33:3060–3069. [PubMed: 14579274]

59. Groves T, Katis P, Madden Z, Manickam K, Ramsden D, et al. In vitro maturation of clonal CD4+CD8+ cell lines in response to TCR engagement. J Immunol. 1995; 154:5011–5022. [PubMed: 7730608]

60. Baillie-Johnson H, Twentyman PR, Fox NE, Walls GA, Workman P, et al. Establishment and characterization of cell lines from patients with lung cancer (predominantly small cell carcinoma). Br J Cancer. 1985; 52:495–504. [PubMed: 2998422]

61. Köhler G, Lefkovits I, Elliott B, Coutinho A. Derivation of hybrids between a thymoma line and spleen cells activated in a mixed leukocyte reaction. Eur J Immunol. 1977; 7:758–761. [PubMed: 563329]
62. Hämmerling GJ. T lymphocyte tissue culture lines produced by cell hybridization. Eur J Immunol. 1977; 7:743–746. [PubMed: 304002]
63. Boyse EA, Itahura E, Stocker CA, Iritan J, Miura M. LyC: a third locus specifying alloantigens exposed only on thymocytes and lymphocytes. Transplantation 11. 1971:3Sl.
64. Nie H, Maika SD, Tucker PW, Gottlieb PD. A role for SATB1, a nuclear matrix association region-binding protein, in the development of CD8SP thymocytes and peripheral T lymphocytes. J Immunol. 2005; 174:4745–4752. [PubMed: 15814699]
65. Liu L, Chahroudi A, Silvestri G, Wernett ME, Kaiser WJ, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nature Med. 2002; 8:185–189. [PubMed: 11821904]
66. Sims RJ 3rd, Weihe EK, Zhu L, O’Malley S, Harriss JV, Gottlieb PD. m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. J Biol Chem. 2002; 277:26524–26529. [PubMed: 12011100]
67. Kuhn JR, Poenie M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. Immunity. 2002; 16:111–21. [PubMed: 11825570]
68. Byrne JA, Oldstone MBA. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus 11. Clearance of virus in vivo. J Virol. 1984; 51:682–689. [PubMed: 6332201]
69. Bai XC, Deng F, Liu AL, Zou ZP, Wang Y, et al. Phospholipase C-gamma1 is required for cell survival in oxidative stress by protein kinase C. Biochem J. 2002; 363:395–401. [PubMed: 11931670]
70. Takayama H, Trenn G, Sitkovsky MV. A novel cytotoxic T lymphocyte activation assay. Optimized conditions for antigen receptor triggered granule enzyme secretion. J Immunol Methods. 1987; 104:183–190. [PubMed: 3500234]
71. Srivastava RK, Sheng Mi Qing, Hardwick JM, Longo DL. Deletion of the Loop Region of Bcl-2 Completely Blocks Paclitaxel-Induced Apoptosis. Proc Natl Acad Sci USA. 1999; 96:3775–3780. [PubMed: 10097113]
72. Liu L, Chahroudi A, Silvestri G, Wernett ME, Kaiser WJ, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nat Med. 2002; 8:185–189. [PubMed: 11821904]
Figure 1. Structure and expression of Smyd1C

A. Schematic of Smyd1 locus and isoforms. Smyd1C’s transcription is initiated from a start site ~160 bp upstream of CD8β. The SET domain is split into S and ET portions by the Mynd domain. Exons 2–11 are common with 3′ UT (smaller white boxes). The unique exon 1 of Smyd1C, light blue; muscle and heart unique exon 1, dark blue; Smyd1A unique exon 5, gray; Smyd1b unique exon 6, orange. B. Smyd1C is expressed strongly in mouse thymocytes and weakly in spleen and lymph nodes. Smyd1C-specific primers were used to amplify Smyd1C cDNA here and in other RT-PCR figures (Table 1) with GAPDH serving as an internal loading control. C. Smyd1C is expressed exclusively in CD8+ T cell lines. Derivation and references for these cell lines is provided in Materials and Methods. CD8 SP or CD8CD4 DP lines are denoted in red. D. Smyd1C is expressed in CD8 SP and CD4CD8 DP thymocytes. cDNA was prepared from magnetically isolated CD4SP, CD8SP, DP and DN C57BL/6 thymocytes and subjected to RT-PCR. E. Expression of Smyd1C is downregulated in response to treatment with CD3 + CD28, Con A or PMA + Ionomycin (P +I). Red cell-deleted, whole thymocytes and splenocytes were cultured with the above stimuli. Cells from each of these conditions were harvested at the hourly time points (indicated only for P+I) and mRNA of Smyd1C was examined by RT-PCR. Data shown are representative of a minimum of 3 independent experiments. F. Smyd1C is expressed most highly in splenocytes following splenocytes, following 6 days of mixed lymphocyte reaction (MLR) using C57BL/6 splenocytes as effectors and irradiated BALB/c splenocytes as targets (details provided in Materials and Methods). G. Confirmation of Smyd1C expression in splenocytes following 6 days stimulation with P+I or MLR by anti-Smyd1C western blotting (faint upper band apparent in some lanes is nonspecific).
Figure 2. Smyd1C partitions within mitochondria and peripheral supramolecular activation clusters of activated CD8 T cells

A. Smyd1C localizes exclusively within the cytoplasm of splenocytes following 6 days P+I stimulation. Nuclei are marked by Hoechst staining. Images here and in subsequent figures were acquired using a 12 bit CCD camera (Model DVC-1312M, DVC, Austin, TX) on a Nikon Diaphot 200 fluorescence microscope. B. Smyd1C localizes to the cytoplasm following transient transfection into non-lymphoid cells, including C2C12 (immortalized myoblasts), 293 T (transformed human embryonic kidney cells) and NIH3T3 (immortalized fibroblasts). C. Smyd1C localizes to mitochondria following P+I stimulation of magnetic-bead purified CD8 splenocytes. Green, anti-Smyd1C; red, the mitochondrial-specific dye, TMRE. D. Smyd1C colocalizes with CD8 at the immunological synapse following 6 days of MLR employing C57BL/6 splenocytes as effectors and irradiated BALB/c splenocytes as targets. Upper panel: Smyd1C co-localizes with CD8 at the interface of stimulated CD8 T cells and APC. Lower panel: Smyd1C localizes at CD8-stimulator-cell peripheral supramolecular activation clusters (pSMACs) as indicated by co-staining with the pSMAC component, LFA-1. Images shown are representative of a minimum of 20 cell pairs.
Figure 3. *Smyd1C* Conditional Knockout (CKO) mice show reduction in thymus and show no differences in T cell subsets nor T cell developmental markers

A. A representative example of *Lck-Cre*-mediated deletion in the thymus and spleen. Total RNA from various tissues was extracted from representative wild-type (WT) and CKO (*Smyd1C*^{Flox/Flox};*Lck-Cre*) C57/BL6 mice. Semi-quantitative RT-PCR was employed for detecting mRNA levels, and GAPDH served as loading control. KO *Smyd1C* expression is reduced ~80% in thymus and spleen (albeit, WT splenic levels are considerably lower). Expression of paralogues *Smyd1A* and B exclusively in skeletal muscle and heart are unaffected by *Smyd1C*CKO.

B. FACS profiles show no difference between *Smyd1C*CKO and WT in CD4CD8 DP nor CD4 or CD8 SP subsets in either thymus (upper panel) or spleen (lower panel).

C. FACS analyses of markers representative of T cell developmental in thymocytes show no change in CKO relative to WT. Upper panels: CKO, red; WT, green; CKO, purple. Lower panel: Equivalent levels of CD3 expression on WT and CKO, indicated for WT as fill (CD4S, green; CD8SP, blue; DN, red) and for CKO as lines (CD4SP, purple; CD8SP, brown; DN, green). Means were established from representative data of 8 mouse pairs.
Figure 4. Activation results in reduction in Smyd1C-deficient CD8+ splenocytes yet T cell activation markers are unchanged

A. Activation-induced CD8 SP T cell loss. CD8+ splenic T cells from WT and CKO mice were incubated with plate-coated anti-CD3 (5 ug/ml) and CD28 (5 ug/ml). At the indicated time points, cells were harvested and stained with anti-CD4 (FITC) and anti-CD8 (PE) antibodies. The numbers in each dot-plot represents the percentage of each cell population. The inset provides the absolute cell numbers from day 5 cultures.

B. Analysis of T cell activation markers in Smyd1C CKO spleens. Histograms showing CD69, CD44 and CD25 expression on gated CD4SP, CD8SP and non-T cells from day 5 cultured cells (panel A) were analyzed by FACS. WT, green; CKO, purple. CD25 and CD69 were up-regulated in gated CKO CD8 cells. Plots are representative data of 10 mouse pairs.
Figure 5. *Smyd1C*-deficient splenocytes undergo activation-induced apoptosis and CTL killing

A. CKO splenocytes proliferate normally as measured by BrdU incorporation. CD3+ CD28-stimulated WT (green) and CKO (purple) splenocytes were tested for proliferation following intracellular incorporation over 12 h with BrdU (10 uM). CD4 and CD8 cells were then isolated and stained with respective antibodies for FACS analyses. The Y axis is plotted as Mean Fluorescence Intensity (MFI); WT, green; CKO, purple.

B. CKO thymocyte proliferation is slightly reduced in response to PMA + ionomycin (P+I) stimulation. Cells were stimulated with ConA, anti (α)-CD3, α-CD3 + α-CD8 or P+I for 3 days and then analyzing for ³H-thymidine incorporation as described in Materials and Methods. *p<0.07.

C. *Smyd1C* CKO cells are deficient in activation-induced apoptosis. CD8 splenocytes were isolated from WT and CKO mice 8 d after infection of mice with LCMV (detailed in Methods and Materials). EL-4 cells were labeled and pulsed with the LCMV peptide NP<sub>396–404</sub> for 1h and then incubated with splenocytes at an EL-4 to splenocyte ration of 25:1) for 2 h. After 30 min incubation with a cell-permeable, fluorogenic caspase substrate, cells were analyzed by flow cytometry. Shown is a representative plot of 3 experimental replicates (p<0.05).

D. Release of BLT esterase following stimulation with P+I for 4 h is impaired in CKO splenocytes. Error bars show averages of 3 representative experiments; **p<0.01.

E. *Smyd1C*-deficient splenocytes actively undergo apoptosis in response to TCR stimulation. Magnetic sorted CD8 cells from WT and CKO mice were incubated with anti-CD3 and anti-CD28 or medium alone. Cells were stained with annexin V and propidium iodide (PI) at the indicated time points and analyzed by FACS. Data shown are representative of 3 independent experiments.

F. Activated *Smyd1C*-deficient CD8+ splenocytes are defective in killing. Cells fractionated in panel A were harvested at 48 h of culture, and whole cell lysates of WT and CKO CD8+ splenocytes were subjected to Western blotting with anti-caspase 3; upper band represents the full length (uncleaved) and the lower band, the cleaved caspase 3 product. All data are shown as means of 4 independent experiments; **p<0.01.
Figure 6. Association of Smyd1C with FKBP38 and Bcl1–2 enhances mitochondrial localization

A, B. Reduced mitochondrial residence of Bcl-2 and FKBP38 in Smyd1C-deficient CD8 T cells. Isolated CD8 splenocytes were stimulated with PMA + Ionomycin (P+I) or anti-CD3+anti-CD28 for 24–48 h. Mitochondria were then co-stained with MitoTracker CMXRos (red), anti-Bcl-2 or anti-FKBP38 (green) and Hoechst (blue). Fluorescent images and overlaps were obtained on Leica SP2 AOB5 or a Nikon Diaphot 200 fluorescence microscopes. Shown are representative images of >100 captured for each panel. Images shown in A and B are representative of a minimum of 20 cell pairs.

C. Interaction of FKBP38 or Bcl-2 with Smyd1C in mammalian cells. NIH3T3 or 293T cells were transiently cotransfected with pBK-CMV-Smyd1C together with either FLAG-FKBP38 or FLAG-Bcl-2. Whole cell lysates (WCL) were immunoprecipitated with anti-Smyd1C and fractionated by SDS-PAGE prior to Western blotting with anti-FLAG mAb. Red circle bands denote immunoprecipitation of corresponding endogenous proteins. WCL representing 5% of the total protein purified served as input for each immunoprecipitation assay; GAPDH served as a loading control.

D. Smyd1C forms a ternary complex with Bcl-2 and FKBP38. NIH3T3 cells were transiently cotransfected with pBK-CMV-Smyd1C and FLAG-Bcl-2 with (+) or without (−) FLAG-FKBP38. E. WCL was immunoprecipitated, fractionated on 12% SDS-PAGE gels, and then immunoblotted with anti-Bcl-2 or anti-FKBP38. Lanes in which designated antibodies were not employed are indicated by (−). Whole cell lysates (WCL) representing 5% of the total...
protein purified served as input for each immunoprecipitation assay; GAPDH served as a loading control.
Figure 7. Smyd1C enhances phosphorylation of Bcl-2 via interaction with and activation of Calmodulin

A. Loss of Smyd1C results in reduction of phosphorylation of Bcl-2. PMA + Ionomycin-activated splenic CD8 T cells were labelled with [32P]orthophosphoric acid, immunoprecipitated (IP) with polyclonal anti-Bcl-2, and fractionated by SDS/PAGE. Following transfer to nylon, half of the filter was exposed to autoradiography (16 hr at −80°C), while the other half was subjected to immunoblotting with a Bcl-2 monoclonal Ab. Shown are representative data of 3 independent experiments.

B. Smyd1C, but not Smyd1A or Smyd1B interacts with Calcineurin (CaN). NIH3T3 cells were co-transfected with FLAG-CaN and either pBK-CMV-Smyd1A, -B or -C. Two days later, WCLs were fractionated by SDS-Page and subjected to Western blotting subsequently probed with anti-CaN or a pan-anti-Smyd1 polyclonal Ab. WCL representing 5% of the total protein purified served as input for each immunoprecipitation assay; GAPDH served as a loading control. Shown are
representative data of 3 independent experiments. C. *Smyd1C* is essential for maximal CaN phosphatase activity. WT and *Smyd1C* CKO CD8+ splenocytes were purified over magnetic beads. WCLs were prepared and then employed for colorimetric determination of CaN phosphatase activity using a phosphopeptide as substrate (detailed in Materials and Methods). Detection of free phosphate release was measured by formation of a complex between malachite green molybdate and free orthophosphate that absorbs at 620 nm. Data shown are the mean concentration of phosphate released measured in four independent experiments; **p<0.001. D. A schematic model by which activated T cell fate is determined by *Smyd1C*. The model postulates that upon CD8 T cell stimulation, activated CaN forms a complex with Bcl-2. Meanwhile, *Smyd1C* is gradually up-regulated and may exert a dominant negative effect by sequestering FKBP38. This results in de-repression of CaN by FKBP38 and induction of dephosphorylation and mitochondrial localization of Bcl-2, resulting in anti-apoptotic action of Bcl-2.