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Dampening of death pathways by Schnurri 2 is essential for T cell development

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Abstract

Generation of a diverse and self-tolerant T cell repertoire requires appropriate interpretation of T cell receptor (TCR) signals by $CD4^+CD8^+$ double positive (DP) thymocytes. Thymocyte cell fate is dictated by the nature of TCR:MHC-peptide interactions, with signals of higher strength leading to death (negative selection) and signals of intermediate strength leading to differentiation (positive selection)[1]. Molecules that regulate T cell development by modulating TCR signal strength have been described but components that specifically define the boundaries between positive and negative selection remain unknown. Here we show that repression of TCR-induced death pathways is critical for proper interpretation of positive selecting signals *in vivo*, and identify Schnurri2 (Shn2) as a crucial death dampener. Our results indicate that $Shn2^{-/-}$ DP thymocytes inappropriately undergo negative selection in response to positive selecting signals, thus leading to disrupted T cell development. $Shn2^{-/-}$ DP thymocytes are more sensitive to TCR-induced death *in vitro* and die in response to positive selection interactions *in vivo*. However, Shn2-deficient thymocytes can be positively selected when TCR-induced death is genetically-ablated. *Shn2* levels increase after TCR stimulation suggesting that integration of multiple TCR:MHC-peptide interactions may fine tune the death threshold. Mechanistically, Shn2 functions downstream of TCR proximal signaling compenents to dampen Bax activation and the mitochondrial death pathway. Our findings uncover a critical regulator of T cell development that controls the balance between death and differentiation.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Contributions

T.L.S. designed the study and performed all experiments; V.L. and D.C.J. contributed to discussions and provided technical assistance; A.J.L. performed preliminary experiments; T.T. and S.I. provided reagents; T.L.S. and L.H.G. wrote the paper.

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Cell intrinsic defects in the generation of T cells generally map to either T cell receptor (TCR) proximal signaling molecules or to two major pathways: the calcineurin/NFAT and Raf/MEK/Erk pathway [2–5]. Schnurri (Shn) family proteins are large zinc-finger containing proteins that regulate morphogenesis in *C. elegans* and *Drosophila*[6,7]. In vertebrates, they influence bone formation, adipogenesis, and memory T cell survival[8–10]. Interestingly, Shn2$^{-/-}$ mice have very few post-selection DP thymocytes or mature single positive (SP) thymocytes but how Shn2 regulates T cell development has remained a mystery (Fig. 1a)[11].

We began investigating this severe defect by examining TCR proximal signaling events that are known to be required for, or associated with, positive selection. Shn2$^{+/+}$ and Shn2$^{-/-}$ DP thymocytes similarly upregulated CD69, a canonical activation marker, and had comparable patterns of tyrosine phosphorylated proteins after TCR stimulation suggesting that TCR proximal signaling is unaltered (Supplementary Fig. 1). Shn2$^{-/-}$ DP thymocytes also had equivalent Erk1/2 phosphorylation after TCR activation (Fig. 1b). In addition, Shn2$^{-/-}$ DP thymocytes showed normal NFATc1 dephosphorylation, NFATc1 nuclear localization, and induction of the NFAT target gene Egr2 in response to TCR stimulation, thus indicating that the calcineurin/NFAT pathway is activated normally in these cells (Fig. 1c, Supplementary Fig. 2, data not shown).

In further support of this, microarray analysis of CD4^{+}CD8^{+}CD69^{-} thymocytes before and after TCR stimulation yielded very few differences in basal and induced gene expression between Shn2$^{+/+}$ and Shn2$^{-/-}$ thymocytes (data not shown). A second, independent microarray analysis also did not detect significant changes in gene expression (T.T. and S.I. unpublished observations). To further examine specific transcription factor activity we transfected luciferase reporter constructs into control and Shn2$^{-/-}$ primary DP thymocytes. NFAT, AP1, NFκB, and Smad activation was comparable between Shn2$^{+/+}$ and Shn2$^{-/-}$ DP thymocytes (Supplementary Fig. 2). While evidence in other cell types suggests that Shn proteins may influence NFκB or Smad activity these pathways are not regulated by Shn2 in thymocytes[8,12].

Based on the unexpected finding that Shn2 does not regulate proximal TCR signaling or positive selection pathways, we examined Shn2$^{-/-}$ DP cells more generally. Thymocyte death *in vivo* can result from either negative selection (in response to strong TCR signals) or death by neglect (in response to weak or no signal)[1]. Therefore, we investigated the response of Shn2$^{-/-}$ DP thymocytes to a gradient of TCR stimulation. Shn2$^{-/-}$ DP thymocytes were strikingly sensitive to death induced by CD3/CD28 ligation, exemplified by a nearly 10-fold shift in responsiveness (Fig. 1d). In contrast, Shn2$^{+/+}$ and Shn2$^{-/-}$ DP thymocytes died comparably in response to CD3 alone, under *in vitro* conditions that approximate death by neglect, and in response to the non-TCR death signals dexamethasone, Fas, or TNF-α treatment (Fig. 1e,f and Supplementary Fig. 3). Thus, while Shn2$^{-/-}$ DP thymocytes respond normally to non-TCR death signals, they appear to be hyper-responsive to TCR/costimulation-induced cell death. Shn2$^{-/-}$ DP thymocytes showed increased levels of early death markers, namely caspase activation and disruption of mitochondrial membrane potential, as well as increased upregulation of the late death marker Annexin V in response to CD3/CD28 stimulation (Fig. 1d,g). Collectively, these data indicate that Shn2$^{-/-}$ DP

thymocytes do not have an inherent survival defect, but rather have an altered perception of TCR signal strength that leads to inappropriate apoptosis via the intrinsic death pathway.

Since TCR-induced death is specifically affected in Shn2$^{-/-}$ DP thymocytes, we examined components upstream of the mitochondria in the intrinsic death pathway to map the stage at which Shn2 functions. In thymocytes, TCR signaling can induce Bim and ultimately lead to conversion of Bax to an active conformation, disruption of the mitochondrial membrane, and apoptosis[13–15]. Using a conformation specific antibody we found increased Bax activation in Shn2$^{-/-}$ DP thymocytes after TCR stimulation, indicating that increased cell death in Shn2$^{-/-}$ DP thymocytes is mediated by this pro-apoptotic protein (Fig. 1h and Supplementary Fig. 4)[16].

Our findings suggesting that Shn2-deficiency alters the threshold of TCR-mediated cell death led us to consider a role for Shn2 in the balance of positive and negative selection. Strong negative selecting signals activate both the differentiation pathway and the death pathway; however, because the death pathway acts in a dominant manner, negative selecting signals result in apoptosis rather than differentiation[2]. On the contrary, signals of intermediate strength are able to induce differentiation and positive selection without triggering activation of the intrinsic cell death pathway. It remains unclear how positive selection prevails in response to signals of intermediate strength. We hypothesized that Shn2 acts to dampen death induced by TCR signaling, thereby allowing positive selection to proceed in response to signals of intermediate strength. In this scenario, Shn2$^{-/-}$ thymocytes inappropriately activate death pathways in response to positive selection signals (Supplementary Fig. 12). Therefore, we asked whether preventing Shn2$^{-/-}$ thymocytes from dying would allow them to complete positive selection and mature into CD4 and CD8 SP cells. The pro-apoptotic protein Bim is a key component of TCR-induced death in thymocytes[17]. We therefore used the Bim$^{-/-}$ genetic background as a tool to eliminate TCR-induced death. Genetic inhibition of negative selection by Bim-deficiency rescued differentiation of Shn2$^{-/-}$ thymocytes, as evidenced by the presence of mature SP cells in the thymus and mature T cells in the periphery (Fig. 2a,b and Supplementary Fig. 5). These data reinforced the conclusion that differentiation pathways are functional in Shn2-deficient cells, which can mature when death pathways are eliminated.

Next we closely analyzed the consequence of positive selection interactions *in vivo* in the presence or absence of Shn2. Our *in vitro* data indicate that in the absence of Shn2 there could be increased death in response to positive selection interactions. However, Shn2$^{+/+}$ and Shn2$^{-/-}$ mice with polyclonal TCR repertoires have similar total thymocyte numbers and an equally low percentage of dying thymocytes. This may result from the fact that in a diverse, polyclonal repertoire only a small fraction of DP thymocytes are undergoing a positive selection at any snapshot in time (Fig. 3b). On the other hand, the majority of DP thymocytes in TCR transgenic (Tg) mice express a TCR of defined specificity; in this system, the effect of positive and negative selecting signals can be dissected and analyzed separately. To examine the role of Shn2 in the interpretation of TCR signals in response to positive selection interactions *in vivo*, we crossed Shn2$^{-/-}$ mice to three different TCR Tg strains: DO11, AND, HY. Shn2$^{-/-}$ DO11 mice had dramatically reduced total and DP thymocyte numbers and an altered CD4/CD8 staining profile (Fig. 3a,b). A change in

DP:DN ratio can be indicative of either a developmental block at the DN stage or increased DP cell death. The decrease in thymocyte number was not due to an early DN cell defect, as DN cell development was normal in Shn2$^{-/-}$ mice (Supplementary Fig. 6). A higher percentage of Shn2$^{-/-}$ DO11 thymocytes were AnnexinV^{+} and showed caspase activation indicating that the decrease in thymic cellularity is due to increased cell death. Hence, we conclude that Shn2 actively represses thymocyte death in response to positive selection interactions *in vivo* (Fig. 3c,d). Results from the AND and the HY TCR Tg models also indicated that in the absence of Shn2 positive selection was converted to death *in vivo*. Shn2$^{-/-}$ AND and Shn2$^{-/-}$ HY female mice had reduced thymocyte number and an altered CD4/CD8 staining profile (Fig. 3e,f and Supplementary Fig. 7). We also used the DO11 TCR Tg system to more closely investigate the rescued SP cells in Shn2$^{-/-}$ Bim$^{-/-}$ mice and determine whether cells could be rescued from death in response to positive selection interactions. The appearance of mature SP thymocytes and large numbers of mature T cells in the periphery in Shn2$^{-/-}$ Bim$^{-/-}$ DO11 TCR Tg mice indicated that Bim-deficiency rescued Shn2$^{-/-}$ thymocytes receiving positive selection interactions *in vivo* (Fig. 3g,h and Supplementary Fig. 8).

We interrogated negative selecting interactions *in vivo* in the absence of Shn2. Shn2-deficiency did not alter negative selection in response to the male specific self antigen in male HY mice or in response to endogenous deleting superantigens in AND mice(Supplementary Fig. 7)[18]. Thus, in the absence of Shn2, physiologic negative selection is unaltered.

To determine whether TCR:MHC interactions were required to kill Shn2$^{-/-}$DO11 thymocytes *in vivo*, bone marrow from Shn2$^{+/+}$DO11 and Shn2$^{-/-}$DO11 mice was transplanted into irradiated MHC I$^{-/-}$II$^{-/-}$ and WT recipients. Thymocyte numbers in Shn2$^{-/-}$DO11→MHC I$^{-/-}$II$^{-/-}$ chimeras were rescued to the level of Shn2$^{+/+}$DO11→MHC I$^{-/-}$II$^{-/-}$chimeras, whereas thymocyte numbers in Shn2$^{-/-}$DO11→WT chimeras were still low (Fig. 4a,b). These data together with the rescue of CD4 SP cells in Shn2$^{-/-}$Bim$^{-/-}$DO11 TCR Tg mice points to a role for positively selecting interactions killing Shn2$^{-/-}$thymocytes *in vivo*. Additionally, naive Shn2$^{-/-}$ DO11 DP thymocytes that developed in MHC I$^{-/-}$II$^{-/-}$ chimeras were more sensitive to death induced by APCs loaded with OVA peptide *in vitro* as evidenced by enhanced death at lower concentrations of peptide (Fig. 4c). Similar results were obtained when Shn2$^{-/-}$ AND DP thymocytes were exposed to their cognate antigen *in vitro* (Fig. 4d).

Interestingly, Shn2 heterozygous mice have only half the normal number of mature SP cells suggesting that thymocyte viability is highly sensitive to Shn gene dosage *in vivo* (Supplemental Fig. 9)[11,19]. Given that thymocytes undergo multiple interactions before being relegated to death or differentiation[20,21], we investigated whether Shn2 expression levels are static or dynamic during thymocyte development. TCR stimulation or pharmacological activation increases *Shn2* expression by DP thymocytes (Fig. 4e–g). Although CsA treatment has a small effect on Shn2 induction, Ca^{2+}/calmodulin blockade completely inhibits Shn2 induction suggesting another calmodulin-dependent pathway regulates TCR-induced Shn2 expression. The dynamic changes in *Shn2* levels suggest that fine tuning of the death threshold may rely on the integration of previous TCR:MHC

interactions and that upregulation of Shn2 expression may serve to adjust death pathway thresholds and allow positive selection of appropriate cells.

During T cell development the proper interpretation of TCR signals is the critical event that induces the differentiation of productive cells and the elimination of potentially destructive ones. There is a clearly defined border between positive and negative selection that is dependent on the affinity of TCR:MHC interactions[22]. Where and how the signals activating positive and negative selection pathways diverge remains unknown. Here we show that Shn2 is critical in maintaining the balance between death and differentiation by repressing TCR-induced death pathways. Investigation of events upstream of Bax activation revealed that basal and TCR-induced levels of Bim and Nur77, two critical regulators of TCR-induced cell death during negative selection,[17],[23] were unperturbed in the absence of Shn2 (Supplementary Fig. 10). In addition, phosporylation of Bim, critical for cell death *in vivo*, was unaltered in Shn2$^{-/-}$ DP thymocytes (Supplementary Fig. 4)[24]. Total protein levels of other apoptotic regulatory molecules (Bcl-2, Bcl-$_{XL}$, Mcl-1, and Bax) were also normal (Supplementary Fig. 10). These data suggested that Shn2 does not simply regulate the balance between established pro-death and anti-death molecules. Considerable effort has focused on uncovering the intricate mechanism of action by which death proteins regulate cell death in many cell types.[25] Shn2 may control activity of these molecules by regulating their associations with one another or affecting other post-translational modifications. Understanding the exact biochemical mechanism by which Shn2 regulates Bax activation may reveal how the intrinsic death pathway is regulated.

Interestingly, while some mature thymocytes do develop in Shn2$^{-/-}$ mice there is a complete absence of T cells in Shn2$^{-/-}$Shn3$^{-/-}$ mice, suggesting a functional redundancy among Shn family members (Supplementary Fig. 11). Based on the studies described herein, we propose a new model in which the previously enigmatic Shn family of proteins functions to oppose activation of negative selection pathways downstream of TCR:MHC interactions of intermediate strength. In this model, Shn-mediated dampening of the intrinsic death pathway is a requisite component to allow the differentiation of mature T cells.

Methods summary

Cell isolation and flow cytometry

Double positive thymocytes were isolated by positive selection of total thymocytes with anti-CD8α magnetic beads (Miltenyi Biotech) with purity greater than 90%. Antibodies used for flow cytometry were from BD Pharmingen and BioLegend. Death was analyzed with FAM Poly Caspases Assay Kit (Molecular Probes) and AnnexinV and JC-1 Mitoscreen Kits (BD Pharmingen) according to manufacturer's protocol.

Cell stimulation

For platebound CD3/28 stimulation, plates were coated overnight with indicated concentrations of antibodies. *In vitro* death assays were performed in triplicate in 96 well flat bottom plates with 10^5 cells per well for 20 hours. Percent live DP was determined by AnnexinV staining and normalized to unstimulated values. Antigen specific stimulation: 5×10^5 DP thymocytes were co-cultured with 5×10^4 APC (M12.4.1 cells[26] for DO11 TCR

Tg and PI39 cells[27] for AND TCR Tg) and indicated concentrations of $OVA_{323-339}$ peptide (GenScript) or PCC_{88-104} peptide (Anaspec) for 20 hours. Percent live DP was determined by AnnexinV staining and normalized to values of M12 or P139 cells alone. For RNA and protein analyses, $0.5–1\times10^7$ cells were stimulated in 6 well plates. For inhibitor studies, cells were stimulated with PMA(5nM), Ionomycin(1μM), or platebound αCD3(10μg/ml)/ αCD28(50μg/ml). Cells were pretreated with inhibitors for 30 min: BAPTA-AM (20μM), CsA (200ng/ml), W7 (100μM), KN62 (10μM), STO69 (10μg/ml), BIM1 (1μM), Go6976 (1μM), UO126 (10μM), and SP600125 (10μM).

Bax IP

Cells (2×10^7) were stimulated with PMA (5nM) and Ionomycin (1μM) for 4 hours, washed once with PBS, and resuspended in lysis buffer (10mM HEPES, 150mM NaCl) containing either 0.2% NP-40 or 1% CHAPs plus protease and phosphatase inhibitors. Active conformation Bax antibody (6A7, eBioscience) was used to immunoprecipitate overnight. Membranes were blotted with rabbit polyclonal Bax antibody (Millipore).

Methods

Mice

Mice were housed in the pathogen-free facility at the Harvard School of Public Health in accordance with guidelines from the Center for Animal Resources and Comparative Medicine at Harvard Medical School, and were used between 4 and 12 weeks of age unless otherwise noted. $Bim^{-/-}$[17], DO11[28], AND[29], HY[18], MHC $I/II^{-/-}$[30], $Shn2^{-/-}$[11], and $Shn3^{-/-}$[9] have been previously described. Bone marrow chimeras were generated as follows: Balb/c or MHC $I/II^{-/-}$ mice were irradiated with 2 doses of 550 rads and transplanted with 5×10^6 total bone marrow cells. Chimeras were analyzed 6–8 weeks post transfer.

Real-time PCR

RNA was isolated using Trizol (Invitrogen) and DNAse treated. A High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to synthesize cDNA. SYBR Green Technology and a Stratagene Mx3005P thermocycler were used for real-time PCR. Values were normalized to β-actin. Sequences of primers: Shn2, 5′-TGAGCAGAGCACAGACACG-3′ and 5′-GGGCTCACTTTGTCAGAAGC-3′; β-actin, 5′-GCTCTGGCTCCTAGCACCAT-3′ and 5′-GCCACCGATCCACACAGAGT-3′; Bim, 5′-CGACAGTCTCAGGAGGAACC -3′ and 5′-CATTTGCAAACACCCTCCTT-3′; Nur77, 5′-TCCTCATCACTGATCGACACG-3′ and 5′-AGCTCTTCCACCCGACGAG-3′

Biochemical Analysis

Western blotting used standard methods and antibodies to: Bim phospho-Thr112 (gift from Roger Davis)[24], Bim phospho-Ser65 (Cell Signaling), phospho-ERK (197G2, Cell Signaling), ERK (Millipore), NFATc1 (7A6, BD Pharmingen), phospho-Tyr (4G10, Upstate), Mcl-1 (Biolegend), Nur77 (12.14, BD Pharmingen), Bim (Stressgen), and SP1, HSP90, $Bcl\text{-}_{XL}$, and Bcl-2 (Santa Cruz). For NFATc1 localiation, nuclear and cytoplasmic purification was performed using the NE-PER kit (Pierce).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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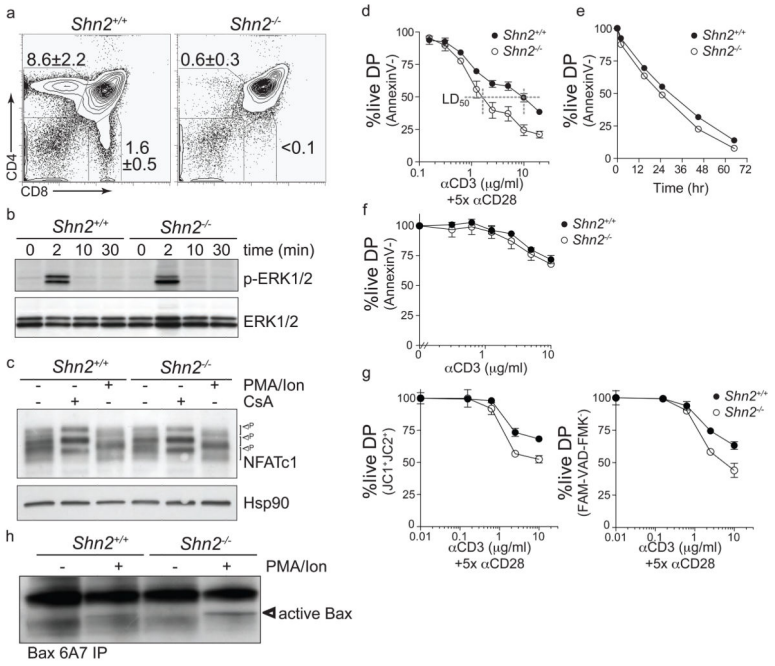


Figure 1. Increased TCR-induced death in Shn2$^{-/-}$ thymocytes

a, Representative plots of total thymocytes (mean ± standard devation, n>10). b, Immunoblot of phosphorylated and total ERK1/2 in DP thymocytes after CD3 crosslinking. c, Immunoblot of NFATc1 in DP thymocytes after PMA/Ion or CsA treatment for 30 min. d, DP thymocytes stimulated with platebound αCD3/αCD28 (d) orαCD3 for 20 hours (f), or left unstimulated for indicated times (e). g, DP thymocytes stimulated with platebound αCD3/αCD28 for 20 hours. Percent live DP determined by JC1/2 or FAM-VAD-FMK staining. h, Immunoblot of total Bax in DP thymocytes after 4 hour PMA/Ion stimulation and immunoprecipitation with 6A7 antibody.

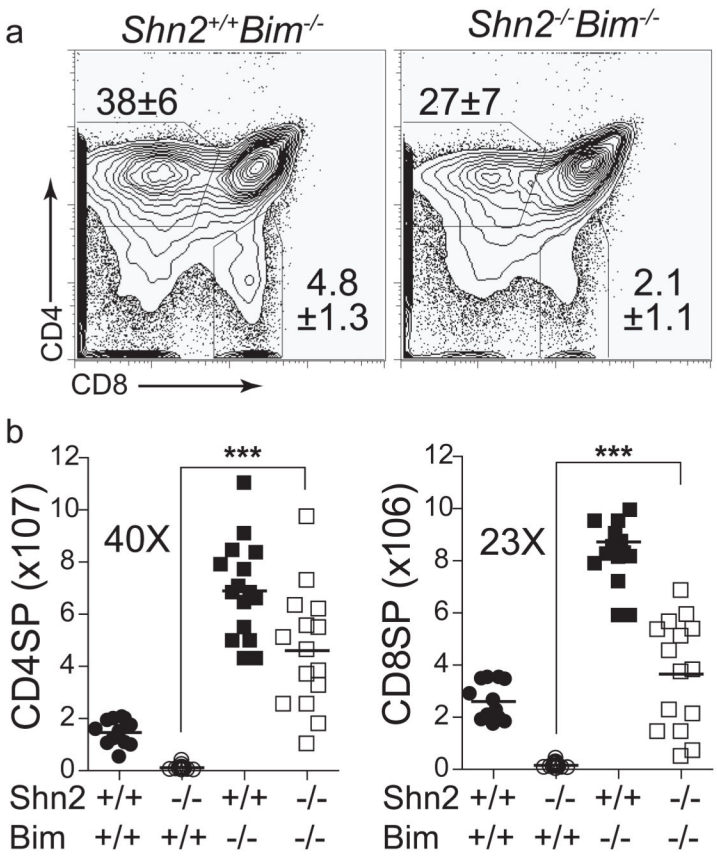
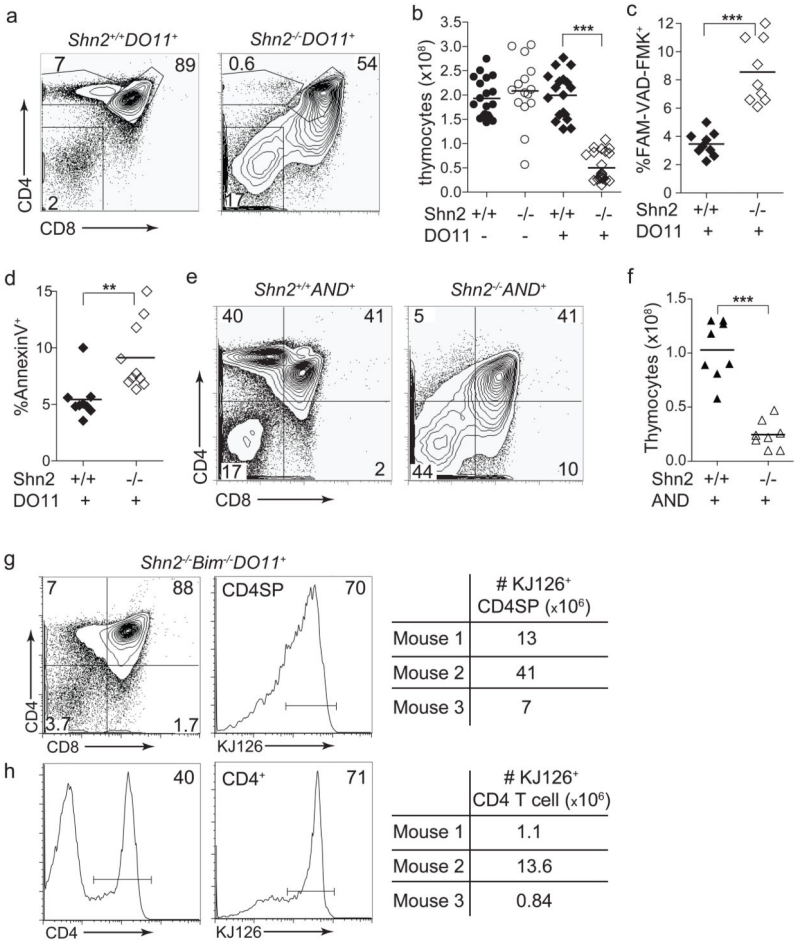


Figure 2. Bim deficiency rescues positive selection in Shn2$^{-/-}$ mice

a, Representative plots of total thymocytes (mean ± standard devation, n>10). b, Total numbers of CD4SP and CD8SP cells from indicated genotypes were calculated to include only mature TCR^{hi} cells. Each dot represents a mouse, bar represents the mean, number indicates fold change ***p<0.0001.



	# KJ126+ CD4SP (x10⁶)
Mouse 1	13
Mouse 2	41
Mouse 3	7

	# KJ126+ CD4 T cell (x10⁶)
Mouse 1	1.1
Mouse 2	13.6
Mouse 3	0.84

Figure 3. Conversion of positive selection to death *in vivo*

a, Representative plots of total thymocytes (n>10). b, Total thymocyte cell numbers. Percent of thymocytes (with CD4SP cells excluded) staining with FAM-VAD-FMK reagent (c) and AnnexinV reagent (d). e, Representative plots of total thymocytes (n=8). f, Total thymocyte cell numbers. Representative plots of thymus (g) and lymph node (h) of $Shn2^{-/-}Bim^{-/-}DO11^{+}$ mice (mouse 3 is shown) with total clonotypic CD4 cell numbers from each mouse shown in the table. For all plots, numbers indicate percent of cells within each gate. For all graphs, each dot represents a mouse, bar represents the mean: ***p<0.0001, **p<0.001.

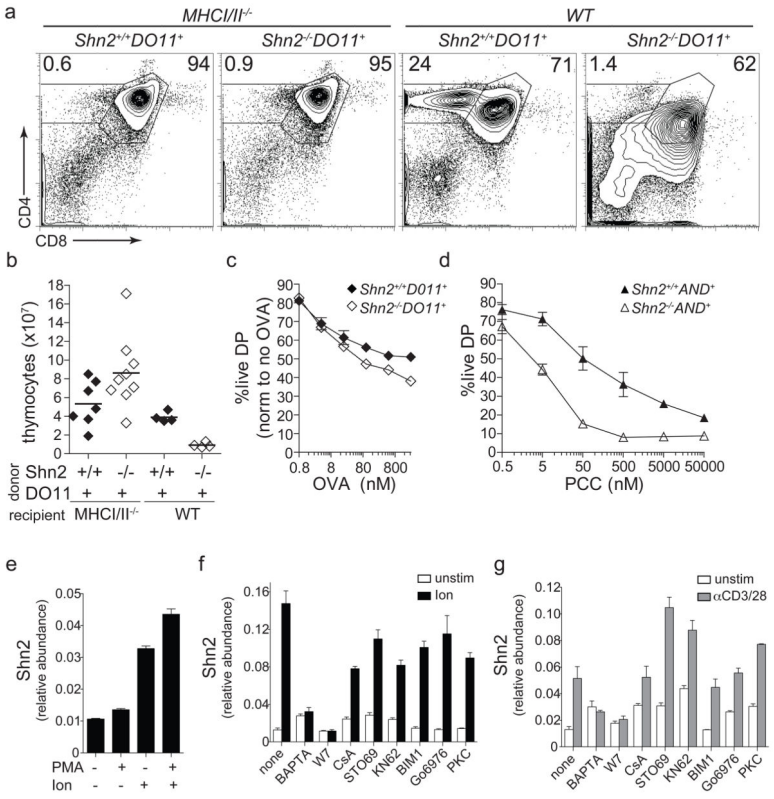


Figure 4. Shn2 dampens TCR-induced death *in vivo*

a, Representative plots of total thymocytes from bone marrow chimeras. Numbers indicate percent of cells within each gate. b, Total thymocyte cell numbers. Each dot represents a mouse, bar represents the mean. c, DP thymocytes stimulated by M12 cells pulsed with OVA peptide for 20 hours. d, DP thymocytes were stimulated by PI39 cells pulsed with PCC peptide for 20 hours. Quantitative PCR analysis of Shn2 expression by DP thymocytes after 3 hour stimulation with PMA and/or Ionomycin (e), Ionomycin (f), or platebound αCD3/αCD28 (g). Cells were pretreated with inhibitors for 30 min as indicated.