

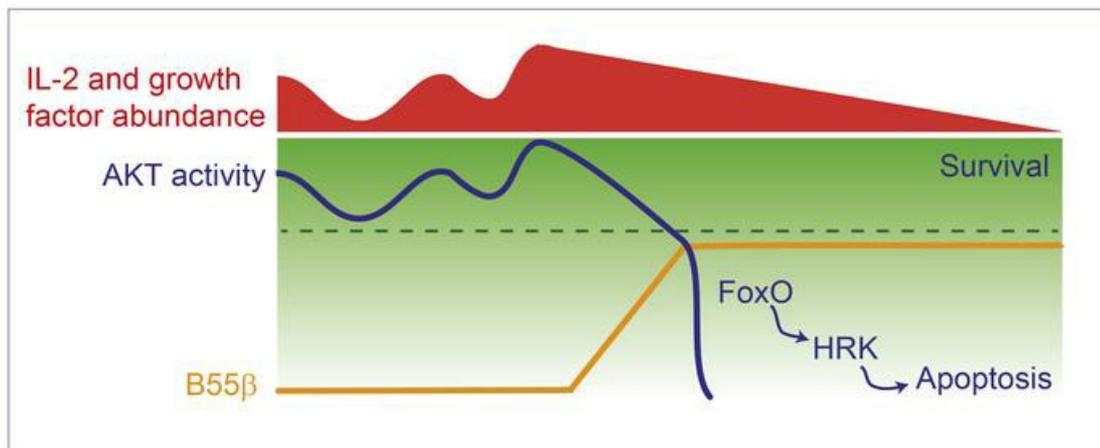
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Noé Rodríguez-Rodríguez, ... , Florencia Rosetti, Jose C. Crispin

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Protein phosphatase 2A B55 β limits CD8 T cell lifespan following cytokine withdrawal

Noé Rodríguez-Rodríguez,^{1,2,3,4} Iris K. Madera-Salcedo,¹ J. Alejandro Cisneros-Segura,¹ H. Benjamín García-González,¹ Sokratis A. Apostolidis,^{3,5} Abril Saint-Martin,¹ Marcela Esquivel-Velázquez,^{1,6} Tran Nguyen,³ Dámaris P. Romero-Rodríguez,⁷ George C. Tsokos,³ Jorge Alcocer-Varela,¹ Florencia Rosetti,^{1,*} and José C. Crispín^{1,8,*}

¹ Departamento de Inmunología y Reumatología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, 14080, Mexico

² Departamento de Inmunología, Instituto de Investigaciones Biomédicas, UNAM, Mexico City, 04510, Mexico

³ Division of Rheumatology, Beth Israel Deaconess Medical Center & Harvard Medical School, Boston, MA, 02215, USA

⁴ Present address: Medical Research Council, Laboratory of Molecular Biology, Cambridge, CB2 0QH, UK

⁵ Present address: Division of Rheumatology, University of Pennsylvania, Philadelphia, PA 19104, USA.

⁶ Present address: Dirección de Investigación, Hospital General de México "Dr. Eduardo Liceaga", Mexico City, 06726, Mexico

⁷ Flow Cytometry Core Facility, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Mexico City, 14080, Mexico

⁸ Tecnológico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Monterrey, NL, 64849, Mexico

Authorship note: NRR and IKMS share the first author position in this work. NRR produced 56% and IKMS 44% of the data presented in the main figures.

* Correspondence:

José C. Crispín

Departamento de Inmunología y Reumatología
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán
Vasco de Quiroga 15
Tlalpan, 14080
Mexico City, Mexico
Phone: +5255-5487-0900 ext. 2610
carlos.crispina@incmnsz.mx

Florencia Rosetti

Departamento de Inmunología y Reumatología
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán
Vasco de Quiroga 15
Tlalpan, 14080
Mexico City, Mexico
Phone: +5255-5487-0900 ext. 2610
florencia.rosettis@incmnsz.mx

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ABSTRACT

How T cells integrate environmental cues into signals that limit the magnitude and length of immune responses is poorly understood. Here, we provide data that demonstrates that B55 β , a regulatory subunit of the phosphatase PP2A, represents a molecular link between cytokine concentration and apoptosis in activated CD8 T cells. Through the modulation of AKT, B55 β induced the expression of the pro-apoptotic molecule Hrk in response to cytokine withdrawal. Accordingly, B55 β and Hrk were both required for in vivo and in vitro contraction of activated CD8 lymphocytes. We show that this process plays a role during clonal contraction, establishment of immune memory, and preservation of peripheral tolerance. This regulatory pathway may represent an unexplored opportunity to end unwanted immune responses, or to promote immune memory.

KEYWORDS

AKT; Apoptosis; B55 β ; cytokine withdrawal; Hrk; *PPP2R2B*; PP2A; T cells.

Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that regulates a large number of cellular processes including apoptosis (1). PP2A is ubiquitously expressed and defects in its activity and/or expression have been linked to cancer (2), neurodegenerative diseases (3), and autoimmunity (4). PP2A, as other serine/threonine phosphatases, is comprised of three subunits: scaffold (PP2A A), catalytic (PP2A C), and regulatory (PP2A B) (5). The scaffold and catalytic subunits assemble into a heterodimer that can associate, in a mutually exclusive manner, to a relatively large number of regulatory subunits. The choice of regulatory subunit determines, to a large extent, the subcellular location and target specificity of the holoenzyme (5).

Altered expression and activity of PP2A C in T cells has been documented in patients with systemic lupus erythematosus (SLE), a chronic autoimmune disease (4). In mice, increased expression of PP2A C in T cells promoted immune-mediated glomerulonephritis (6), whereas regulatory T cell (Treg)-specific PP2A A deficiency annulled Treg suppressive function and caused early onset systemic autoimmunity (7). Thus, the correct function of PP2A is of utmost importance for T cell function.

Indirect evidence suggests that the B55 β regulatory subunit of PP2A controls death and survival in different cell lineages. Expansion of a CAG repeat in the 5' region of *PPP2R2B*, the gene that encodes B55 β , causes type 12 spinocerebellar ataxia (8). The pathogenic repeat has been proposed to increase the expression of B55 β (9) and cause disease by facilitating neuronal death (10, 11). On the other hand, decreased B55 β expression, caused by promoter hypermethylation, has been linked to several types of cancer (12–14). In patients with SLE, defective expression of B55 β in activated T cells is associated with resistance to apoptosis (15, 16). Therefore, although little is known about the function(s) of B55 β in normal cell physiology, data produced in different experimental and clinical settings indicates that its expression facilitates cell death whereas its absence may allow abnormal cell survival and transformation.

Apoptosis represents the main mechanism responsible for the elimination of activated T cell clones during immune responses (17). Accordingly, defects in molecules that regulate the extrinsic and intrinsic pathways of apoptosis result in immune responses of excessive magnitude or length (18). During acute immune responses, T cell survival relies on environmental cues, including antigen and cytokine concentrations, but how these extrinsic elements, or their absence, trigger apoptosis is unknown. Here, we identify B55 β as an essential molecule that controls T cell survival during the termination of immune responses and show that, in human and mouse cells, it regulates apoptosis through a conserved pathway that culminates in the induction of the pro-apoptotic molecule Hrk.

RESULTS

B55 β controls the lifespan of activated CD8 T cells

Defective expression of the B55 β regulatory subunit of PP2A is associated with resistance to cytokine-withdrawal induced death (CWID) in activated T cells from patients with SLE (15). To determine the role of B55 β in the immune response, and in particular to define its participation in the regulation of apoptosis of activated T cells, we used a *Ppp2r2b* conditional knockout (cKO) mouse from the KOMP repository to generate a T cell-specific B55 β cKO in the B6 background (*Ppp2r2b^{fl/fl} Cd4.Cre⁺*).

T cell-specific B55 β deficiency did not affect T cell ontogeny or thymocyte populations and peripheral T cell subsets were numerically normal in 6-week-old cKO mice (Supplemental Figure 1A). However, as cKO mice aged, they exhibited a gradual decrease in the frequency of naïve (CD44⁺CD62L⁺) T cells and a reciprocal increase in CD44⁺CD62L⁺ central memory (CM) T cells. No consistent differences in the abundance of CD44⁺CD62L⁻ effector memory (EM) T cells were observed (Supplemental Figures 2 and 3). The accumulation of CM T cells was mainly due to the presence of increased numbers of CM CD8 T cells. Interestingly, aged mice accumulated a large amount of IFN- γ -producing CD8 (WT 24.9 \pm 1.6 vs. cKO 39.1 \pm 3.4, p=0.003) and CD4 (WT 3.7 \pm 0.4 vs. cKO 6.2 \pm 0.6, p=0.005) T cells (Supplemental Figure 3).

To define the role of B55 β during an immune response, we generated B55 β -deficient OT-I mice (*Ppp2r2b^{fl/fl} Cd4.Cre⁺ OT-I*). We isolated CD8 T cells from OT-I cKO and WT (*Ppp2r2b^{+/+} Cd4.Cre⁺ OT-I*) mice, adoptively transferred them into CD45.1⁺ recipients, and infected them with ovalbumin-expressing recombinant *Listeria monocytogenes* (LM-OVA). At day 2, there were no differences in the number of c.f.u. of *L. monocytogenes* isolated from the livers of mice that had received WT and cKO cells (WT 585 \pm 235 vs. cKO 358 \pm 111, p=0.415), suggesting that deficiency of B55 β does not affect the effector function of CD8 T cells. At day 7, numbers of WT and cKO OT-I cells were similar, suggesting that B55 β deficiency does not affect CD8 T cell expansion (Figure 1A). To confirm this, we adoptively transferred

CD45.1/2 WT and CD45.2 cKO OT-I cells, in a 1:1 ratio, into CD45.1 recipient mice and infected them with LM-OVA. At day 4 post-infection (p.i.) EdU incorporation confirmed that antigen-induced proliferation is not affected by absence of B55 β (Supplemental Figure 4). In contrast to what was observed during clonal expansion, the number of cKO cells was significantly higher during the contraction phase of the immune response. OT-I cKO cells were 2-fold more abundant at day 14 (1.29 ± 0.11 M vs. 2.76 ± 0.10 M, $p=0.0003$) and 5-fold more abundant at day 30 (0.4 ± 0.11 M vs. 2.0 ± 0.30 M, $p=0.0008$) p.i. than the number of WT OT-I cells (Figure 1A).

B55 β deficiency did not alter the distribution of naïve and activated/memory CD8 T cells during the acute infection. As shown in Figure 1B, naïve OT-I T cells virtually disappeared by day 7 p.i. and were replaced mostly by EM cells. The frequency of the latter ebbed and, at day 30, CM cells represented the most abundant OT-I cell subset in the spleens of infected mice.

Absence of B55 β caused an accumulation of EM and CM cells, but the effect was more marked in CM cells (Figure 1C). This was not explained by different kinetics, because the contraction slope of WT EM and CM cells was similar (EM -3.6 vs. CM -3.3) (Supplemental Figure 5A). When WT OT-I cells were adoptively transferred, only 12.7 and 3.2% of the EM cells present at day 7 p.i. were found at days 14 and 30, respectively. Deficiency of B55 β significantly increased the numbers of EM cells at days 14 (26.6%, $p=0.001$) and 30 (11.3%, $p=0.001$), but the contraction slope was still steep (Figure 1C and Supplemental Figure 5B). The fraction of WT CM T cells found at days 14 and 30 p.i. was 42.1 and 15.6%, respectively. In the case of cKO OT-I cells, the number of CM cells found at days 14 and 30 p.i. was not different than the number of CM cells found at the peak of the response (day 7) when considered as absolute (day 14: 1.3 ± 0.1 M vs. 1.15 ± 0.1 M, $p=0.19$; day 30: 1.3 ± 0.1 M vs. 1.27 ± 0.6 M, $p=0.91$), or relative numbers (day 14: 100% vs. 88.5%; day 30: 100% vs. 97.9%) (Figure 1C). Accordingly, contraction of cKO CM OT-I cells was negligible (Slope 0.03) and markedly different from the kinetics of WT CM cells (day 14 $p=0.004$; day 30 $p=0.001$) (Figure 1C and Supplemental Figure 5).

Analysis of expression of CD127 (IL-7R α) and killer cell lectin-like receptor subfamily G member 1 (KLRG1) in WT and cKO OT-I T cells showed that at day 7 p.i. there were no differences in the abundance of CD127⁺ KLRG1⁻ memory precursor cells or CD127⁻ KLRG1⁺ short-lived effector cells (Figure 1D). However, at days 14 and 30, the numbers of both populations were significantly higher within B55 β -deficient cKO OT-I cells (Figure 1E).

We quantified IFN- γ -producing capacity in WT and cKO OT-I cells upon ex vivo restimulation with their cognate antigen, an OVA-derived peptide (SIINFEKL). As shown in Figures 1F and G, at day 7 the percentage (as well as the absolute number) of cells able to produce IFN- γ was similar in WT and cKO OT-I cells. However, at days 14 and 30, a higher percentage of cKO cells were IFN- γ ⁺ (Figure 1G) and these differences were reflected in ~3-fold (0.39 ± 0.06 M vs. 1.28 ± 0.09 M, $p=0.0004$) and ~9-fold (0.1 ± 0.04 M vs. 0.98 ± 0.18 M, $p=0.0007$) increases in splenic IFN- γ -producing OT-I cells at days 14 and 30, respectively, within cKO OT-I cells when compared to their WT counterparts (Figure 1F).

Collectively, these results suggest that B55 β regulates the lifespan of activated CD8 T cells, in particular of CM CD8 T cells. This role is exerted during steady state and at the resolution of an acute immune response triggered by an intracellular bacterium. Moreover, these results indicate that the accumulation of activated CD8 T cells caused by the absence of B55 β is not caused by an immune response of increased magnitude or by prolonged antigen persistence, but probably to decreased clonal contraction.

B55 β is necessary for cytokine withdrawal-induced apoptosis

Our results, along with previously published work that associated defective B55 β expression with resistance to CWID (15, 16), compelled us to analyze whether increased survival of activated CD8 T cells was caused by defective apoptosis. To this end, we quantified apoptosis in adoptively transferred WT and cKO OT-I cells, 30 days after infection with LM-OVA. As shown in Figures 2A and B, the percentage

of OT-I cells that exhibited apoptotic features was significantly decreased in cKO cells as compared to their WT counterparts (WT $18.7 \pm 3.8\%$ vs. cKO $6.7 \pm 1.3\%$, $p=0.033$). In concordance, caspase 3 activation was significantly lower in OT-I T cells deficient in B55 β (WT $7.4 \pm 0.8\%$ vs. cKO $5.6 \pm 0.4\%$, $p=0.032$) when assessed 14 days after infection with LM-OVA (Figure 2C).

Clearance of activated T cells at the termination of acute immune responses is a complex process in which several molecules that regulate the mitochondrial pathway of apoptosis have been involved (19, 20). Loss of antigen, regulatory T (Treg) cell function, and cytokine withdrawal are considered key elements that trigger the demise of activated T cells (18). To determine whether B55 β deficiency impairs CWID, we activated polyclonal T cells from WT or cKO mice in vitro with anti-CD3 and anti-CD28 and expanded them in the presence of IL-2. After 10 days, cells were replated in fresh full RPMI devoid of IL-2, in the presence of a neutralizing anti-IL-2 antibody, to induce CWID (21). Induction of apoptosis was significantly decreased in B55 β -deficient T cells (WT $36.7 \pm 1.0\%$ vs. cKO $25.6 \pm 1.5\%$, $p<0.001$) (Figures 2D and E). As in the in vivo setting, the consequences of B55 β absence were more marked in CD8 (WT $23.7 \pm 0.6\%$ vs. cKO $15.4 \pm 1.3\%$, $p<0.001$), than in CD4 T cells (WT $14.6 \pm 1.1\%$ vs. cKO $9.4 \pm 1.2\%$, $p<0.05$). Because FoxP3⁺ Treg cells contribute, through IL-2 deprivation, to CD8 clonal contraction (22), we analyzed whether B55 β deficiency could also affect Treg cell kinetics during LM-OVA infection. We observed a modest, albeit statistically significant, increment in Treg cell frequency in mice at days 7 and 30 following infection with LM-OVA in B55 β cKO mice. No differences in absolute Treg cell numbers were observed, suggesting that increased Treg cell frequency represents a compensatory mechanism triggered by failed CD8 clonal contraction (Supplemental Figure 6).

We generated lentiviral particles encoding short hairpin RNAs (shRNA) to silence the expression of B55 β (*PPP2R2B*) in human T cells. T cells from healthy subjects were activated in vitro with OKT3 and anti-CD28 and infected with lentiviruses encoding B55 β -specific (B55 β -sh) or a scrambled control shRNA (Scr-sh) (Supplemental Figure 7). After 10 days of expansion, T cells were deprived of IL-2 and apoptosis

was quantified in effectively infected (mCherry⁺) cells. As shown in Figures 2F and G, B55 β silencing in human T cells significantly decreased apoptosis in total T cells (Scr-sh 7.5 \pm 1.4% vs. B55 β -sh 4.9 \pm 0.4%, p<0.01), CD4⁺ T cells (Scr-sh 3.5 \pm 0.2% vs. B55 β -sh 2.6 \pm 0.1%, p<0.05), and CD8⁺ T cells (Scr-sh 8.5 \pm 1.5% vs. B55 β -sh 4.3 \pm 0.5%, p<0.001). These results indicate that B55 β is necessary for human and mouse T cells to undergo apoptosis in response to cytokine withdrawal and suggest that the accumulation of activated CD8 T cells in the B55 β -deficient animal is due to the abnormal survival of activated clones.

IL-2 withdrawal induces AKT dephosphorylation in a B55 β -dependent manner

The AKT-mTOR signaling pathway is an ancient system that allows cells to adapt to changes in their environment, in particular fluctuations in nutrients and growth factors (23). PP2A regulates the activity of the AKT-mTOR pathway at different levels (24) and in many cell lineages, including T cells (7). Because CWID represents a cellular response to a decline in the concentration of a T cell growth factor, we hypothesized that B55 β triggers apoptosis through the modulation of the anti-apoptotic function of AKT (25–27).

We activated and expanded T cells in vitro and then analyzed AKT phosphorylation in two residues that determine its activity (S473 and T308) (28, 29). Both residues of AKT were phosphorylated in activated T cells, but lost their phosphorylation 4 hours after IL-2 withdrawal (Figures 3A and B). Serum deprivation causes AKT dephosphorylation and apoptosis in different cell lineages. To determine whether in vitro IL-2 withdrawal behaves in an analogous manner to serum deprivation, we analyzed the kinetics of dephosphorylation of AKT and other members of the AKT-mTOR pathway in activated T cells subjected to serum starvation or to IL-2 deprivation. As observed in other cell lineages, serum starvation induced rapid and profound dephosphorylation of the pathway (30, 31). Deprivation of IL-2 induced a similar

response, but changes were only observed after a 4 hour time lag (Figure 3C) that corresponds to the upregulation of B55 β induced by IL-2 withdrawal (15).

The kinetics of AKT dephosphorylation and B55 β induction during cytokine withdrawal suggested that B55 β might mediate AKT inactivation. To test this hypothesis, we compared AKT dephosphorylation in response to IL-2 withdrawal in T cells from WT and cKO mice. As shown in Figures 3D and E, AKT dephosphorylation at 4 and 24 hours was significantly diminished in B55 β -deficient T cells (4 h: WT 0.3 ± 0.06 vs. cKO 0.6 ± 0.02 , $p=0.02$; 24 h WT 0.3 ± 0.08 vs. cKO 0.8 ± 0.14 , $p=0.006$). Next, we analyzed AKT phosphorylation in human T cells after B55 β knockdown. As expected, IL-2 deprivation caused AKT dephosphorylation in cells infected with a control lentivirus. This effect was significantly prevented by B55 β silencing (Figure 3F). When analyzed as MFI (Figure 3G) or as percentage of pAKT⁺ cells (Figure 3H), B55 β knockdown ameliorated AKT dephosphorylation in a statistically significant manner. Over-expression of B55 β in 293T cells caused a significant drop in pAKT along with a reciprocal increase in the abundance of total AKT (Figure 3I-K) that could represent a compensatory mechanism to decreased AKT activity. Interestingly, T cells deprived of IL-2 exhibit an increase in the levels of AKT at the mRNA level (Figure 4D), not reflected by changes at the protein level (Figures 3A and D). This suggests that, as described for other genes, AKT is actively regulated at the translational level in activated T cells (32).

AKT inhibits apoptosis triggered by B55 β

To determine whether the modulation of AKT phosphorylation is required for cell death to occur in response to B55 β , we analyzed apoptosis in response to B55 β forced expression. To this end, human T cells were activated and infected with control or B55 β -encoding lentiviruses and apoptosis was quantified by flow cytometry (Figure 4A). Annexin V binding was detected in a large fraction of the cells after infection with the B55 β -encoding lentivirus (control 19.1 ± 4.4 vs. B55 β 53.4 ± 5.1 , $p=0.007$) (Figure 4B). To test the role of AKT in B55 β -induced apoptosis, we infected T cells with B55 β /mCherry and either

control GFP or AKT/GFP-encoding lentiviruses and analyzed apoptosis in mCherry/GFP co-expressing cells. As shown in Figure 4C, forced expression of AKT tended to decrease B55 β -induced cell death (B55 β 28.25 \pm 5.8 vs. B55 β + AKT 16.7 \pm 5.8, p=n.s.). In humans and mice, AKT has three isoforms (AKT1, AKT2, and AKT3) encoded by three highly homologous genes (33). We analyzed the expression of the three genes in CD4 and CD8 T cells and found that *AKT2* and *AKT3* are the predominant isoforms in resting cells (Figure 4D). However, during T cell activation and expansion, *AKT2* and *AKT3* are downregulated and the three isoforms are transcribed at roughly the same level. IL-2 deprivation stimulates the transcription of *AKT2* and *AKT3*, and 24 hours following IL-2 deprivation, *AKT2* is the most highly expressed isoform in CD4 and CD8 T cells (Figure 4D). *AKT2* silencing significantly increased the rate of apoptosis induced by cytokine withdrawal (Figure 4E), and *AKT2* (but not *AKT1*) levels significantly correlated with apoptosis rate in cells deprived of IL-2 ($r=0.64$, $p=0.03$; Supplemental Figure 8). These results suggest that AKT2 may represent the AKT isoform that promotes T cell survival during growth factor withdrawal. Taken together, these data indicate that B55 β induced during IL-2 withdrawal promotes AKT dephosphorylation which hampers the pro-survival capacity of AKT and triggers apoptosis.

B55 β promotes FoxO transcriptional activity

Forkhead box O (FoxO) transcription factors regulate several cellular processes including apoptosis (34). Phosphorylation by AKT (35, 36) inhibits the transcriptional activity of FoxO factors by promoting their association with 14-3-3 proteins and consequently their sequestration in the cytoplasm (37).

To determine whether AKT inactivation in response to B55 β expression modifies the regulation of FoxO factors, we cotransfected MCF-7 cells with a plasmid encoding a GFP-FoxO1 fusion protein (38) and either an mCherry control, or an mCherry/B55 β -encoding plasmid (Figure 5A). FoxO1 localized mainly in the cytoplasm and was found in the nucleus of only ~20% of the cells transfected with a control plasmid.

In contrast, cotransfection of B55 β caused FoxO1 nuclear translocation in a majority of the cells (control 20.9 ± 5.6 vs. B55 β 76.4 ± 9.6 , $p=0.007$, Figure 5B).

Next, we infected human T cells with control or B55 β -encoding lentiviruses and measured the expression of FoxO-regulated genes by qPCR. To this end, we chose *BCL2L11* (Bim), *PMAIP1* (Noxa), *BBC3* (PUMA), *HRK*, *BCL2*, and *BCL2L1* (Bcl-x_L), because they are regulated by FoxO factors and/or involved in apoptosis (39, 40). As shown in Figure 5C, forced expression of B55 β in T cells, specifically induced the transcription of *BBC3* (p53 upregulated modulator of apoptosis; PUMA) and *HRK* (Harakiri) in a significant manner. Transcription of other apoptosis-related genes (i.e. *BIM*, *PMAIP1*, *BCL2*, and *BCL2L1*) was not consistently modified by the ectopic expression of B55 β in T cells.

B55 β is required for the upregulation of *Hrk* during cytokine withdrawal

Hrk (Harakiri or Dp5) is a pro-apoptotic Bcl-2-homolog 3 (BH3)-only protein of the Bcl-2 family (41). *Hrk* was first identified by differential display screening in neurons undergoing apoptosis induced by neuronal growth factor deprivation (42). More recent work determined that HRK is necessary for apoptosis of diffuse large B cell lymphoma cells in response to inhibition of the PI3K-AKT-FoxO1 survival pathway (43, 44). Because expression of Hrk was induced by forced expression of B55 β (Figure 5C), we hypothesized that it may represent the main pro-apoptotic molecule responsible for the cell death-inducing effects of B55 β . To test whether B55 β is necessary for Hrk induction during CWID, we subjected activated T cells from WT and cKO mice to cytokine withdrawal. As expected, IL-2 deprivation caused an upregulation of Bim (*Bcl2l11*), Puma (*Pmaip1*), Noxa (*Bbc3*), *Bcl2*, and Hrk. Absence of B55 β was associated with a modest, non-significant reduction in the expression of Bim, Puma, Noxa, Bcl2, and Bcl-x_L at 6 and 24 hours after IL-2 deprivation. In contrast, B55 β deficiency abolished the upregulation of *Hrk* at 6 (WT 6.2 ± 1.8 vs. cKO 1.2 ± 0.9 , $p=0.035$) and 24 hours (WT 31.1 ± 8.6 vs. cKO 8.2 ± 2.5 , $p=0.018$) (Figure 6A). To assess the relevance of these findings in the in vivo system, we adoptively transferred

WT and cKO OT-I cells into CD45.1⁺ mice and infected them with LM-OVA. At days 7 and 15, we sorted the transferred CD45.2⁺ OT-I cells and analyzed the expression of Bim, Puma, Noxa, and Hrk. As shown in Figure 6B, upregulation of the four pro-apoptotic genes was detected at day 15 p.i. in WT cells. Although the kinetics of expression of Bim, Puma, and Noxa were maintained in the absence of B55 β , induction of Hrk was completely abolished in cKO cells.

The mitochondrial pathway of apoptosis is a complex process that involves several proteins. To weigh the individual importance of Hrk, we evaluated the effect of its silencing in cells subjected to CWID. As shown in Figure 6C, Hrk knockdown significantly decreased apoptosis induced by IL-2 deprivation in CD8⁺ and in CD8⁻ T cells. To test the in vivo significance of these findings, OT-I T cells infected with either a control lentivirus encoding a blue fluorescent protein (Cerulean) or a B55 β -specific shRNA (plus GFP) were mixed in a 1:1 ratio and adoptively transferred into RIP-mOVA mice. After 14 days, the mice were sacrificed and the survival of the transferred cells was compared. Cells infected with the control virus were undetectable in most mice. In contrast, *Hrk* knockdown was associated with a significant increase in the number of live transferred cells (Control shRNA 990 \pm 677 vs. *Hrk* shRNA 8813 \pm 3839, p=0.039; Figure 6D).

Taken together, these results indicate that Hrk plays an essential and non-redundant role in the induction of CD8 T cell apoptosis during cytokine withdrawal. Thus, declining IL-2 abundance promotes the transcription of B55 β , AKT regulation, and FoxO transcriptional activation that culminates with Hrk production and apoptosis.

Signaling through AKT2 inhibits B55 β expression

Deficient expression of *Ppp2r2b* could alter IL-2 signaling, conferring resistance to CWID. To examine this possibility, first we compared the expression levels of CD25, the alpha subunit of the IL-2 receptor. As shown in Figure 7A, WT and cKO T cells displayed comparable levels of CD25. Likewise, the response

of WT and cKO cells to IL-2, measured as STAT5 phosphorylation, was also similar in the absence of B55 β (Figure 7B). Finally, the kinetics of STAT5 phosphorylation during IL-2 deprivation were also normal in cKO T cells (Figure 7C). These data indicate that proximal signaling through the IL-2 receptor is not affected by B55 β deficiency.

IL-2 signaling activates at least three pathways: JAK1/3-STAT5, PI3K-AKT-mTORC1, and MAP kinases (45). To determine whether one of these pathways is primarily involved in the regulation of T cell survival during low cytokine conditions, we dissected their contribution to B55 β induction and cell death. IL-7 and IL-15, two cytokines that signal through the common γ_c chain receptor (γ_c) and JAK3, rescued T cells from apoptosis induced by IL-2 withdrawal (Figure 7D), confirming that signaling through γ_c is necessary for activated T cell survival (46). Accordingly, exposure to Tofacitinib, a JAK1/3 inhibitor, induced apoptosis in the presence of IL-2 and promoted the transcription of *PPP2R2B* at comparable levels than IL-2 withdrawal (Figure 7E). Specific inhibition of STAT5 did not cause cell death, nor *PPP2R2B* transcription (Figure 7E), and a comparable lack of effect was observed when PI3K, mTORC1, MEK, p38, and ERK were inhibited using a pharmacologic approach (Figures 7F and G). Addition of the AKT inhibitor, MK-2206, caused inconsistent results. In some experiments, MK-2206 induced cell death in the presence of IL-2, whereas in other experiments, it had no effect (Figure 7G). MK-2206 exerts a differential effect on each AKT isoform (47) and the variations observed could be caused by differential inhibition of the different AKT isoforms. To explore this possibility, we silenced *AKT1* or *AKT2* and quantified B55 β transcription. As shown in Figure 7H, *AKT1* knockdown had no effects on *PPP2R2B* mRNA abundance. In contrast, *AKT2* knockdown was associated with a significant increase in B55 β expression. These data, which are consistent with the increase in apoptosis observed in T cells following *AKT2* silencing (Figure 4E), suggest that AKT2 signaling promotes activated T cell survival during cytokine withdrawal by inhibiting B55 β expression.

B55 β curbs the pathogenic capacity of CD8 T cells

Defects in the contraction of activated T cells can contribute to immunopathology and autoimmune disease. To determine the relevance of the B55 β -regulated apoptotic pathway, we differentiated OT-I CTLs and transferred them into mice that express OVA in the pancreatic β cells (RIP-mOVA). Absence of B55 β was associated with severe pancreatic islet inflammation and early development of diabetes in 80% of the animals. In contrast, B55 β -sufficient cells caused mild inflammation and diabetes only in 20% of the mice (Figures 8A-D). To determine whether the heightened pathogenicity of B55 β cKO T cells could be attributed to enhanced effector function, we compared proliferation, granzyme B expression, degranulation, and cytotoxic capacity between WT and cKO CD8 T cells. As shown in Supplemental Figure 9, we found no differences in these parameters, suggesting that the increase in tissue damage derived from their enhanced survival. In support of this hypothesis, we found that the survival of cKO OT-I CTLs transferred to RIP-mOVA mice was significantly higher than the survival of WT OT-I CTLs (Figures 8E and F).

To assess the relevance of the B55 β -associated increase in CD8 T cell survival, we transferred CD45.2 OT-I cells (WT or cKO) into CD45.1 mice and infected them with LM-OVA. Thirty days later, mice were inoculated with a melanoma cell line that expresses OVA (B16.OVA) and survival and tumor growth were monitored (Figure 8G). As shown in Figure 8H to K, cKO OT-I cells dealt with the OVA-expressing melanoma in a significantly more efficient manner. Mice that received cKO cells survived more (43% vs. 10% survival at Day 50, $p=0.008$, Figure 8H) and tumor growth was importantly retarded (Figures 8I-K). Because lack of B55 β does not affect the effector function of CD8 T cells, the heightened response to OVA-bearing tumor cells reflects a more robust memory response associated with increased CD8 memory T cell lifespan.

DISCUSSION

The factors that determine when an activated T cell will terminate its functional life and activate the mechanisms that will lead to its apoptotic death remain poorly understood. Elements external to the cell, such as abundance of cytokines and growth factors, persistence of antigenic stimulation, and the action of regulatory T cells have been identified as triggers that precipitate apoptosis in activated T cells through the mitochondrial pathway (17, 20). The importance of each of these factors has been demonstrated by experimental manipulation. For example, genetic deletion of Bim, decrease the rate of clonal contraction during acute infections (48). How these external factors are integrated by the cell and translated into a death signal has remained unknown (49). Our results introduce B55 β as a key player in this process.

Expression and function of B55 β is intimately associated with the pathways that sense the external milieu, in particular abundance of nutrients and growth factors, and implement customized metabolic adaptations. Transcription of B55 β is set in motion by reduced AKT2 activity and its main effect is to terminate AKT activity. Thus, presence of B55 β may signal commitment to apoptosis and a transition to a state where pro-survival factors can no longer rescue the cell. AKT activity may fluctuate in response to variations in the frequency and intensity of the stimuli that it senses. It is possible that when such fluctuations reach the threshold where B55 β is elicited, its AKT-regulating function may inhibit further teetering and, by shutting off the pathway, actively terminate the pro-survival capacities of AKT, block further AKT activation, and initiate apoptosis (Figure 9). How different AKT-activating stimuli differentially affect AKT function in T cells is unknown and warrants further investigation. Our combined in vivo and in vitro approaches were not designed to dissect the contribution of the different cues that modulate AKT activity. By depriving activated T cells from IL-2, we were able to observe B55 β expression followed by the events that culminate in cell death. However, at this point we ignore the relative importance of decreased cytokine availability in the complex in vivo setting. The fact that B55 β deficiency prolongs the lifespan of antigen-specific CD8 T cells and inhibits the expression of Hrk, indicates that our in vitro system recapitulates to some extent the events that trigger cell death in vivo, but as any reductionist

approach, it may lack depth and complexity when compared to the phenomenon occurring in a natural setting.

AKT represents a family of three highly homologous kinases (AKT1 or PKB α , AKT2 or PKB β , and AKT3 or PKB γ) (33, 50) that play an essential role as pro-survival kinases in different situations and in different cell lineages including healthy (51) and transformed B cells (43). Transgenic expression of myristoylated (constitutively active) AKT1 is associated with increased survival of thymocytes and peripheral T cells during cytokine withdrawal (52, 53). On the other hand, the three isoforms have been shown to promote thymocyte survival in mice with one or more AKT genes deleted, suggesting that the three AKT kinases may exert partially redundant pro-survival activities in immature and mature T cells (54, 55). Our data offer a glimpse at the complexity of this system, as they show that the three isoforms are dynamically regulated during quiescence, cell cycle, and cytokine withdrawal. They further support AKT2 as a major contributor to cell survival during cytokine deprivation, but further studies are required to determine the exact contribution of each AKT isoform to this process and to determine whether B55 β affects the activity of all or individual AKT isoforms.

The regulation of the mitochondrial pathway of apoptosis is a complex process where several cellular stresses (e.g. cytokine deprivation, DNA damage) converge to trigger death (56). This pathway is mainly controlled by interactions between Bcl-2 and a family of related proteins. Cellular stresses promote the expression of pro-apoptotic BH3-only proteins (i.e. Bim, Bad, Puma, Noxa, Hrk) that neutralize Bcl-2 and other anti-apoptotic proteins (e.g. Bcl-x_L) allowing the activation and oligomerization of Bax and Bak which form pores on the mitochondrial outer membrane (57). The functions of BH3-only proteins are redundant and loss of individual molecules tend to have mild consequences (57). Different cellular stresses induce different BH3-only proteins. For example, DNA damage is associated with expression and activation of Puma and Noxa (58, 59). Using over-expression and knockout systems, we have shown that Hrk is induced in T cells by cytokine deprivation in a B55 β -dependent manner. Hrk was first shown to play a pro-apoptotic role in neurons deprived of NGF (42). Later work suggested that Hrk was also induced by

growth factor withdrawal in hematopoietic precursors (60) but this notion was later challenged by findings in an Hrk-deficient mouse (61). Our data demonstrate that in activated T cells, B55 β expression robustly induces Hrk transcription. Further, we demonstrate that B55 β is necessary for Hrk transcription in response to cytokine withdrawal and that this BH3-only protein plays a non-redundant role during T cell CWID. These results are in concordance with Chen et al., who observed Hrk expression in B cell blasts treated with inhibitors of the AKT pathway (43).

As reported in mice deficient in other molecules involved in the regulation of the mitochondrial apoptosis pathway (58, 59, 62, 63), absence of B55 β did not cause a strong spontaneous phenotype. Intriguingly, it was primarily associated with the accumulation of activated CD8 T cells that express CD62L and IFN- γ . The apparent differences in the effects that B55 β deficiency exerts on CD4 and CD8 T cells may be caused by intrinsic differences in apoptosis sensitivity, by different cell expansion and contraction kinetics, or by the fact that CD4 and CD8 T cells are not equally susceptible to IL-2 deprivation caused by Treg cells (22). T cell activation is associated with downregulation of B55 β (15), but resting memory CD8 T cells express higher levels of B55 β than their naïve counterparts (16). Upregulation of B55 β could render memory CD8 T cells particularly susceptible to apoptosis induced by growth factor withdrawal, whilst the abundance of other pro- and anti-apoptotic molecules could modulate their sensitivity to apoptosis. For instance, differential amounts of Bcl-2 and Bcl- χ L in effector and central memory T cells (64), or differences in Bim expression and in the regulation of protective autophagy (65) may render CM T cells more resistant to B55 β -mediated death in the setting of CWID. Increased abundance of IL-7 and IL-15 receptors on memory T cells and precursors may promote the survival of these cells by inhibiting B55 β expression through γ_c signaling. These aspects emphasize the fact that B55 β acts in a cellular context where other unknown factors may modulate its expression, function, and downstream effects. For example, a recent work from our group described how chronic inflammation confers T cells resistant to apoptosis in patients with systemic autoimmune diseases by inhibiting the transcription of B55 β through an epigenetic mechanism (16).

Adoptive transfer of activated and expanded CD8 T cells (CTLs) into mice that express their cognate antigen in the pancreatic β cells was pathogenic only in the absence of B55 β . This effect did not depend on the acquisition of better effector capacities but on the prolonged lifespan conferred by B55 β deficiency. This model is relevant, because it demonstrates that abnormal survival of activated T cells can lead to a breach in tolerance in otherwise healthy organisms. It is also relevant in the setting of cancer immunotherapy where the ability of transferred effector cells depends on their capacity to survive in an environment where cytokines are relatively scarce (66). B55 β silencing or deletion might represent an effective means to overcome this problem, because other solutions, such as administration of exogenous IL-2, have demonstrated only limited success and important secondary effects, such as IL-2-induced toxicity and regulatory T cell expansion (66). The fact that B55 β deficiency allowed antigen-specific CD8 T cells to confer enhanced protection against melanoma, further supports the notion that inhibition of this pathway may allow longer-lived T cell immune responses which may prove useful in certain situations.

In summary, our work demonstrates that the PP2A regulatory subunit B55 β represents a molecular link that commits activated T cells to apoptosis in response to cytokine deprivation through a conserved pathway that involves AKT, FoxO, and HRK. The understanding of this molecular mechanism could have broad implications in our capacity to modulate the length of immune responses in the setting of autoimmunity, cancer, and vaccination.

METHODS

Antibodies, Reagents, Cell lines, Plasmids

Detailed information of plasmids, antibodies, and reagents is provided in Supplemental Tables 1 to 3. LeGo plasmids (67) were a generous gift from Dr. Boris Fehse (University of Hamburg, Hamburg, Germany). pWPI, psPAX2, and pMD2.G plasmids were a generous gift from Dr. Didier Trono (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland). GFP-FoxO1 plasmid was a generous gift from Dr. Domenico Accili (Columbia University, New York, US). MCF-7 and HEK293T/17 cell lines were purchased from ATCC. B16-F10/OVA cells were a generous gift from Dr. Laura Bonifaz. Cells were maintained at 37°C, 5% v/v CO₂, in a humidified incubator in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 200 U/mL penicillin-streptomycin.

Animal Models

Mice used in this study were housed in specific-pathogen-free conditions on a 12 hour light/dark cycle. Mice had ad libitum access to food and water throughout the study. No previous procedure had been performed in the animals. Mice were randomly assigned to the experimental groups. The *Ppp2r2b^{fl/fl}* mouse strain used in this research project was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). CD8⁺ T cells from OT-I *Ppp2r2b^{fl/fl}.Cd4-Cre* or *Ppp2r2b^{+/+}.Cd4-Cre* mice were isolated using CD8α⁺ T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions.

Infection with Listeria. 1x10⁶ cells were injected i.v. into the lateral tail vein of CD45.1 recipient mice. One-day after, 10⁴ c.f.u. of *Listeria monocytogenes* expressing recombinant OVA (LM-OVA; a generous gift from Dr. Michael J. Bevan, University of Washington) were injected i.v (68).

At the indicated times after infection, splenocytes were isolated and analyzed using flow cytometry. To determine cytokine production, 5×10^6 splenocytes were cultured at 37°C for 5 h in complete RPMI 1640 medium with $1 \mu\text{L}/\text{mL}$ Brefeldin A (BD) and stimulated with SIINFEKL ($1 \mu\text{g}/\text{mL}$). After culture, cells were harvested and stained using BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD). All FACS data were acquired using a BD LSR Fortessa and analyzed using FlowJo software (TreeStar). To determine bacterial load in infected mice, animals were sacrificed and perfused with 10 mL of cold PBS. Livers were removed and mashed in 1% saponin. Dilutions were plated on brain-heart infusion agar supplemented with 10 mg/mL of chloramphenicol and grown for 2 days at 37°C (69).

Induction of diabetes. Spleen cells from *Ppp2r2b*^{+/+} OT-I (WT) or *Ppp2r2b*^{fl/fl} OT-I (cKO) mice were differentiated to cytotoxic T lymphocytes (CTL), after RBC lysis, in the presence of 0.5 $\mu\text{g}/\text{mL}$ of SIINFEKL and 100 U/mL of rhIL-2 for 4 days (culture medium and IL-2 were replenished every other day). On day 4, CD8⁺ T cells were isolated by negative selection, and 2.5×10^5 CD8⁺ CTL were adoptively transferred into the lateral tail vein of RIPmOVA mice. Blood glucose was measured every other day and mice were euthanized after two consecutive readings of blood glucose ≥ 250 mg/dL. Pancreata were collected, fixed in 10% formaldehyde, and prepared for histological analyses.

Memory response. After WT or cKO OT-I cell adoptive transfer, CD45.1 mice were infected with LM-OVA as detailed above. Thirty days later, mice received a subcutaneous injection of OVA-expressing B16 melanoma cells (2×10^5). Animals were monitored every 2 days. Tumor size was calculated by using the following formula: tumor size (mm^2) = (length) \times (width) $\times \pi$. Mice with tumor $\geq 800 \text{ mm}^2$ were sacrificed.

In vitro expansion of T cells and induction of cytokine-withdrawal induced death

Human cells. We adapted a protocol published by Snow et al., 2009. T cells were isolated from peripheral blood mononuclear cells using magnetic beads and stimulated in RPMI 1640 medium containing 10% of FBS, 2 mM L-glutamine, and 200 U/mL penicillin-streptomycin. T cell activation was performed with plate-bound anti-CD3 (5 μ g/mL) and anti-CD28 (2.5 μ g/mL). After 72 hours, cells were transferred into new plates, RPMI was duplicated and rhIL-2 (100 U/mL) was added. RPMI and rhIL2 were duplicated every 48 hours. At day 10 of cell culture, T cells were washed in cold PBS and re-plated in complete RPMI without IL-2. At the indicated times, cells were stained for apoptosis quantification, or lysed in TRIZol to obtain RNA.

Murine cells. Cells were isolated from axillary and inguinal lymph nodes from *Ppp2r2b^{fl/fl}.Cd4-Cre* or *Ppp2r2b^{+/+}.Cd4-Cre* mice and as previously indicated for human cells. T cell activation was performed with plate-bound anti-CD3 (2 μ g/mL) and anti-CD28 (2 μ g/mL). At day 10 of cell culture, T cells were washed in cold PBS and re-plated in complete RPMI without IL-2, in the presence of anti-mouse IL-2 (5 μ g/mL). At the indicated times, cells were stained for apoptosis quantification, or lysed in TRIZol to obtain RNA.

Flow cytometry

All in vitro experiments were performed in triplicate for each condition, and the presented data are representative of at least two independent experiments. To assess apoptosis, human or mouse T cells were washed in cold PBS and stained with fluorescently-labeled antibodies (i.e. CD3, CD4, CD8) during 15 minutes. Then, cells were washed and resuspended in Annexin V binding buffer (Biolegend) containing Annexin V and 5 nM Sytox Orange (Thermo Fisher) 10 minutes later, they were analyzed by flow cytometry. In some experiments, caspase activation

was evaluated, using the FAM-FLICA assay kit (Immunochemistry technologies), following the manufacturer's instructions. Briefly, cells were stained with Ghost Dye Red 780 (Tonbo biosciences) in ice-cold PBS for 15 min followed by surface cell staining with the appropriate antibodies. Next, cells were incubated with the FLICA reagent by 30 min at 37°C. After washing, cells were incubated in cold PBS with Sytox Orange (ThermoFisher Scientific) for at least 20 min and acquired in the flow cytometer. For pSTAT5 quantification, cells were washed in cold PBS, fixed for 10 minutes in 1.6% formalin in cell culture RPMI and fixed overnight at -20°C in 90% methanol. Next, cells were washed twice and stained for 45 minutes at RT with a dilution of 8:100 of Alexa Fluor® 647 Mouse Anti-Stat5 (Clone 47/Stat5 (pY694)) before a final wash and acquisition by flow cytometry.

CTL cytotoxic activity

Spleen cells from WT and cKO OT-I mice were differentiated to cytotoxic T lymphocytes (CTL), after red blood cell lysis, in the presence of 0.5 µg/mL of SIINFEKL and 100 U/mL of rhIL-2 for 7 days (culture medium and IL-2 were replenished every other day). At day 7, CD8⁺ T cells were isolated by negative selection for their use. Spleen cells from C57BL/6 mice served as stimulating or target cells. They were pulsed (or not) with 1 µg/mL of SIINFEKL for 1 hour at 37°C. For granzyme B production, CTL were incubated overnight with pulsed spleen cells and Brefeldin A (5 µg/mL) was added during the final 5 hours of culture. Then, cells were stained with the cytofix/cytoperm kit. To determine CTL degranulation, CD107a cell surface expression was assessed. To this end, CTL were incubated for 5 hours with either control or pulsed cells in the presence of anti-mouse CD107a (1:250 dilution), Monesin (2 µM) and Brefeldin A. After 5 hours, cells were surface-stained to identify OT-I (Vα2⁺Vβ5⁺) CD8⁺ T cells. For the in vitro

cytotoxicity assay, unpulsed and pulsed target cells were labeled with different concentrations of cell trace far red (CTFR): 0.1 and 1 μ M, respectively. CTL (effector cells) were incubated at different ratios with 75000 target cells for 5 hours, and CD3⁺CD4⁺CD8⁻ target cell death was measured by Sytox Orange staining.

Gene silencing

Specific shRNA sequences (Supplemental Table 4) were designed using the Broad Institute GPP Web Portal and cloned into LeGO-G or LeGO-Cer. Lentiviral particles were generated in HEK293T/17 cells by cotransfecting the expression plasmid, along with psPAX2 and pMD2.G packaging- and envelope-encoding plasmids. Purified T cells were activated in vitro as detailed previously. Next day, cells were infected with gene-specific (or control) shRNA-encoding lentiviruses in the presence of polybrene (3 μ g/mL). Gene silencing was corroborated by qPCR in sorted cells.

Real-time PCR

Total RNA for real-time PCR was extracted and purified using TRIzol. Reverse transcription was performed in 1 μ g of total using SCRIPT Reverse Transcriptase (Jena Bioscience), following the manufacturer's instructions using oligo-dT primers. Real-time PCR was performed with SYBR Green (Thermo Fisher) labeling in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. mRNA expression was normalized against β -actin. Primers used in this study are listed in Supplemental Table 5.

Western Blotting

Cells were lysed in RIPA buffer (15 μ L of buffer per million cells) during 20 min on ice. Next they were centrifuged for 10 min at 4°C (15,000 *g*) and the supernatant was collected and frozen (-80°C). Cell lysates were thawed on ice and protein concentration was determined using Quick-start Bradford Dye (Bio-Rad). Normalized lysates were denatured by boiling in Laemmli buffer and separated in SDS-PAGE. Proteins were transferred to a PVDF membrane (Thermo Fisher) and detection was done using the indicated primary antibodies, followed by peroxidase-coupled secondary antibodies (Pierce). Proteins were visualized by chemiluminescence (Pierce ECL Western blotting substrate).

Production of lentiviral particles and cell transduction

HEK293T/17 cells were plated in 10 cm Petri dishes in full DMEM. When ~70% confluent, culture medium was replaced with Opti-MEM I (Gibco) and transfected with 10 μ g of shRNA-encoding plasmids or cDNA-encoding plasmids plus 3.5 μ g of MD2.G and 6.5 μ g of psPAX2 using Lipofectamine 2000 (Thermo Fisher). Twenty-four hours after transfection, culture medium was replaced with fresh full DMEM. Lentivirus-containing medium was harvested at 48 and 72 hours after transfection, concentrated using polyethylene glycol (Sigma), and stored at -80°C until use. Human T cells were isolated and activated as described before, and lentiviral particles were added at Day 1 of in vitro activation in the presence of Polibrene (Sigma-Aldrich, 3 μ g/mL). Efficiency of infection was evaluated using flow cytometry and was always between 40 and 75%.

Evaluation of FoxO1 nuclear translocation

MCF-7 cells were plated in 24 well plates in full DMEM. When they reached 70% confluence, they were transfected with a plasmid encoding a FoxO1-GFP fusion protein (38) plus pLVX-EF1 α -IRES-mCherry (control) or pLVX-EF1 α -*PPP2R2B*-IRES-mCherry. Twenty four hours later, membrane-permeable Hoechst 33342 (Thermo Fisher, 100 ng/mL) was added and cells were imaged in an Eclipse TS100 inverted fluorescence microscope (Nikon). One hundred co-transfected cells were quantified per well. Each experiment was performed in triplicate.

Statistics

Statistical tests were calculated using Microsoft Excel and GraphPad Prism. The statistical test used and p values are indicated in each figure. In general, for comparison between two groups, two-tailed paired or non-paired Student's t test was used. The number of times each experiment was repeated is indicated in each figure. Data are presented as means \pm SD or SEM (indicated in each figure). p values less than 0.05 were considered statistically significant. Normal distributions were assumed and no specific method was used to determine whether the data met assumptions of the statistical approach used. No randomization and/or stratification method was applied.

Study Approval

The study was approved by the Research and Ethics Committees of the INCMNSZ (IRE-1805). All participants signed informed consent forms. All experiments involving mice were done in accordance with the Guide for the Care and Use of Laboratory Animals. The Animal Care and Use Committees of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán

(INCMNSZ; IRE-1805) and of the Beth Israel Deaconess Medical Center approved all the performed procedures.

AUTHOR CONTRIBUTIONS

Conceptualization, N.R.R., I.K.M.S., S.A.A., F.R.S. and J.C.C.; Methodology, N.R.R., I.K.M.S., S.A.A., F.R.S. and J.C.C.; Investigation, N.R.R., I.K.M.S., J.A.C.S., H.B.G.G., S.A.A., A.S.M., M.E.V., T.N., D.P.R.R, and J.C.C.; Resources, G.C.T. and J.A.V.; Writing –Original Draft, J.C.C.; Writing –Review & Editing, N.R.R., J.A.C.S., S.A.A., G.C.T., J.A.V., F.R.S. and J.C.C; Funding Acquisition J.A.V., G.C.T., F.R.S. and J.C.C. Supervision, F.R.S. and J.C.C.

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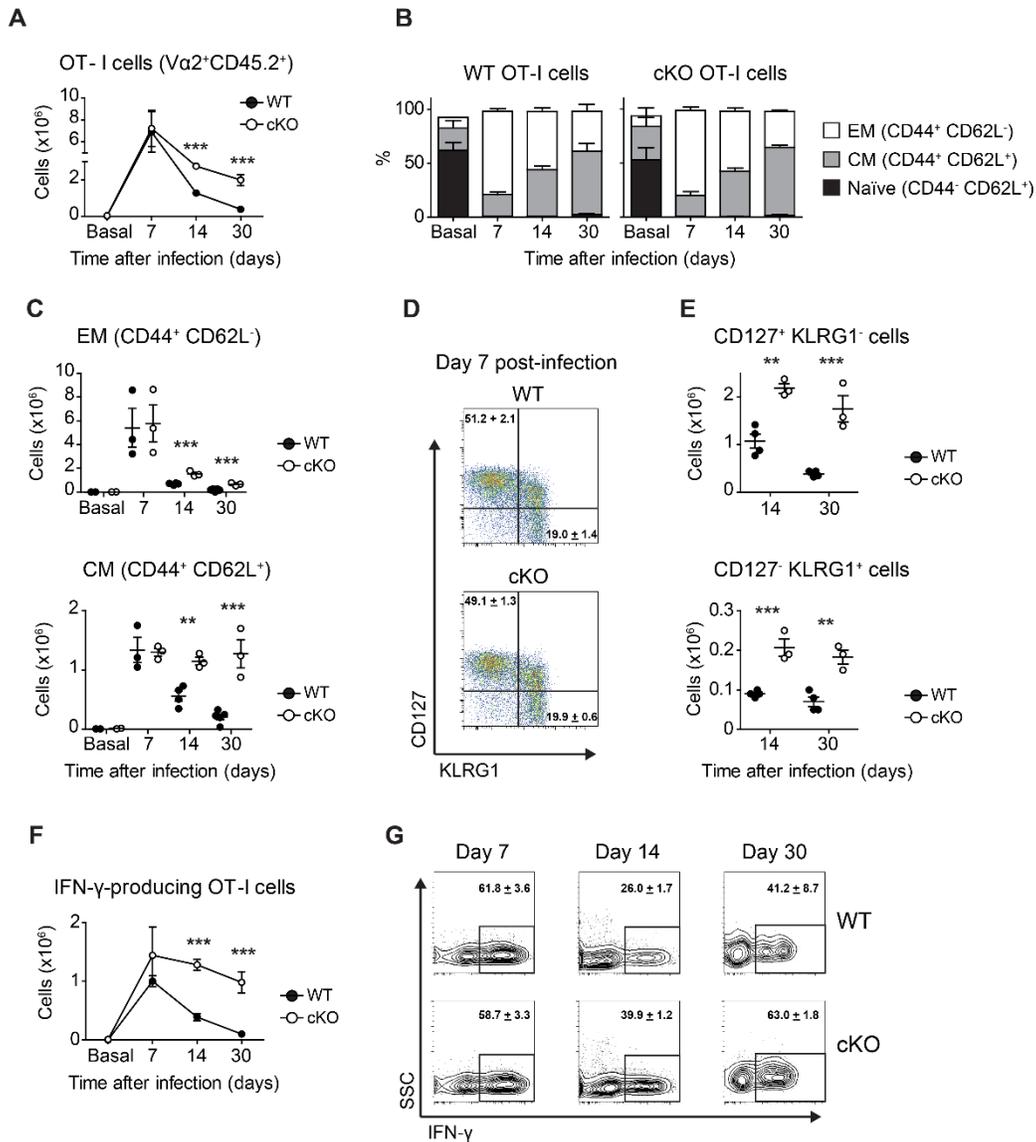


Figure 1. B55 β regulates survival of activated T cells. 10⁶ OT-I CD45.2⁺ *Ppp2r2b*^{+/+} *Cd4.Cre*⁺ (WT) or *Ppp2r2b*^{fl/fl} *Cd4.Cre*⁺ (cKO) cells were adoptively transferred into wild-type CD45.1⁺ mice. Next day, 10⁴ c.f.u. of ovalbumin-expressing *Listeria monocytogenes* (LM-OVA) were i.v. injected into the recipient mice. **(A)** OT-I cells (CD45.2⁺ CD8⁺ Va2⁺ V β 5⁺) quantified in the spleens of recipient mice before infection (Basal) and at the indicated time points. **(B)** Frequency of naïve (CD44⁻ CD62L⁺), effector memory (EM, CD44⁺ CD62L⁻), and central memory (CM, CD44⁺ CD62L⁺) cells within donor-derived OT-I cells. **(C)** OT-I EM and CM cell numbers in spleens of recipient mice quantified at the indicated time points. Each symbol represents a mouse. Means and SEM are indicated by horizontal lines. **(D)** Representative dot plots of CD127 (IL-7R α) and KLRG1 expression on adoptively transferred OT-I cells at day 7 after infection with LM-OVA. Numbers in the dot plots represent the mean \pm SEM of the indicated populations. **(E)** Absolute numbers of OT-I CD127⁺ KLRG1⁻ and CD127⁻ KLRG1⁺ cells in spleens of recipient mice. **(F)** Spleen cells from infected mice, stimulated ex vivo with SIINFEKL, in the presence of Brefeldin A. Results are expressed as absolute numbers of IFN- γ -producing OT-I T cells (mean \pm SEM). **(G)** Representative contour plots from spleen cells stimulated with SIINFEKL (gated in CD45.2⁺ CD8⁺ Va2⁺ V β 5⁺ donor-derived OT-I cells). Numbers represent means \pm SEM of the IFN- γ positive populations. Results from one (out of three) representative experiment (n = 3-5 mice/group) are shown **(A-G)**. For comparison of the means, unpaired two-tailed t tests were used in **(A)**, **(C)**, **(E)**, and **(F)**; ** p \leq 0.01, *** p \leq 0.001.

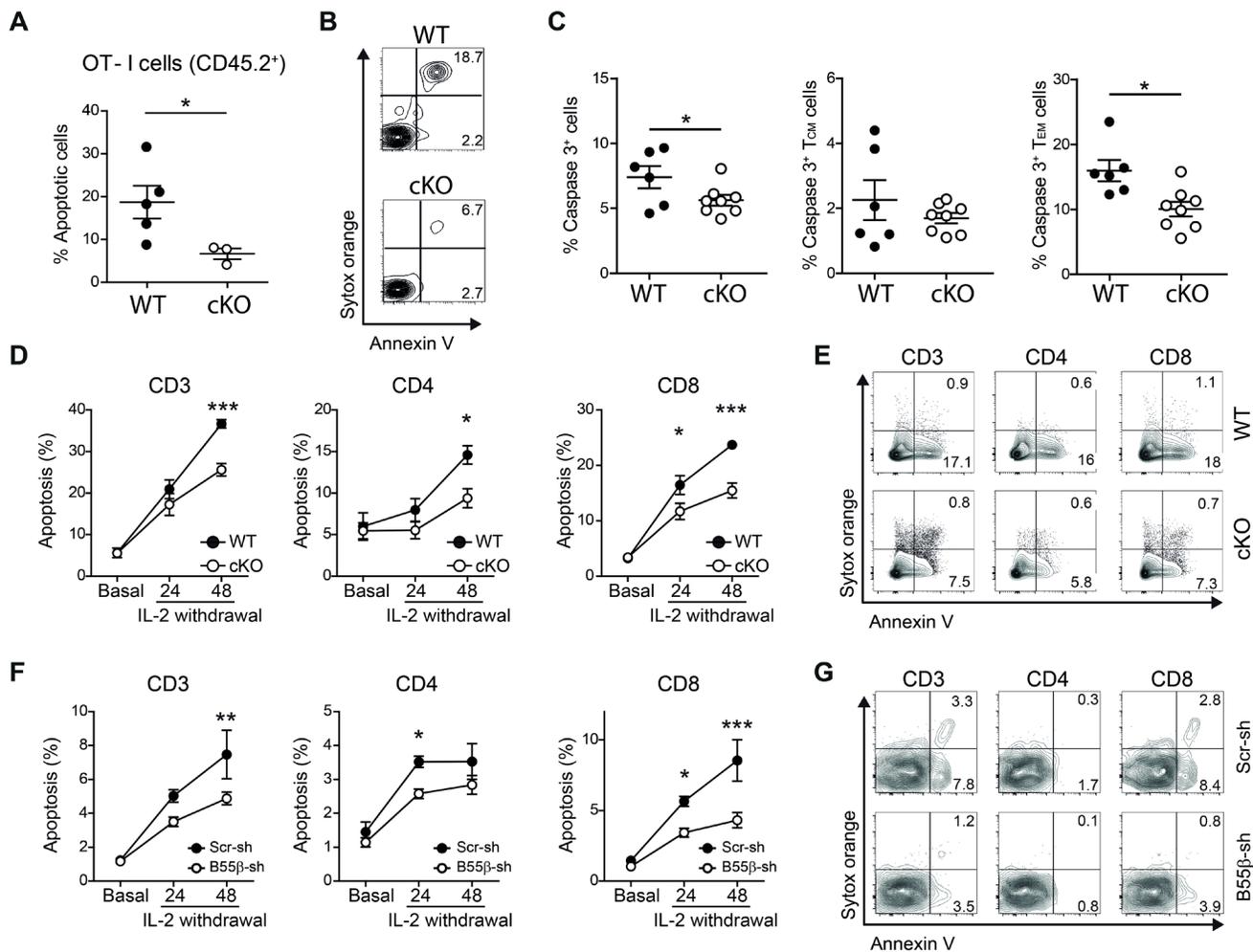


Figure 2. B55β is necessary for cytokine withdrawal-induced death. (A-C) OT-I CD45.2⁺ *Ppp2r2b*^{+/+} (WT) or *Ppp2r2b*^{fl/fl} (cKO) cells were adoptively transferred into CD45.1⁺ mice. Next day, LM-OVA was injected. After 30 (A-B) or 14 days (C), the percentage of apoptotic OT-I cells (CD45.2⁺ CD8⁺ Vα2⁺ Vβ5⁺) was quantified as Annexin V binding (A-B) or caspase 3 activation (C) in spleens. Cumulative data from two experiments ($n=3-8$ mice/group) are shown in (A) and (C). * $p \leq 0.05$, unpaired two-tailed t test. Representative contour plots of OT-I cells (B). Numbers represent mean of the indicated populations. (D-E) T cells from *Ppp2r2b*^{+/+} *Cd4.Cre*⁺ (WT) or *Ppp2r2b*^{fl/fl} *Cd4.Cre*⁺ (cKO) mice were stimulated in vitro with anti-CD3 and anti-CD28 (2 μg/mL). Fresh RPMI and IL-2 (100 U/mL) were replenished every 48 h. At day 10, cells were counted and resuspended in fresh RPMI (10⁶ cells per mL) devoid of IL-2, in the presence of a neutralizing anti-IL-2 antibody (5 μg/mL). Apoptosis was quantified before (Basal) and after 24 and 48 hours of cytokine withdrawal. Results are presented as mean ± SEM. Cumulative results from three independent experiments ($n=2-4$ mice/group/experiment) are shown. * $p \leq 0.05$, *** $p \leq 0.001$ (two-way ANOVA with Bonferroni's posttest). Representative contour plots of WT and cKO cells, 48 hours after IL-2 withdrawal (E). (F) Human T cells were activated and infected in vitro as detailed in (Supplemental Figure 7). Apoptosis was quantified after IL-2 withdrawal in lentiviral-infected cells (mCherry⁺) at the indicated time points. Results are presented as mean ± SEM. Cumulative results from four experiments ($n=1$ /experiment). * $p \leq 0.05$, ** $p \leq 0.01$ *** $p \leq 0.001$ (two-way ANOVA with Bonferroni's posttest). (G) Representative contour plots, at 48 hours after IL-2 deprivation are shown.

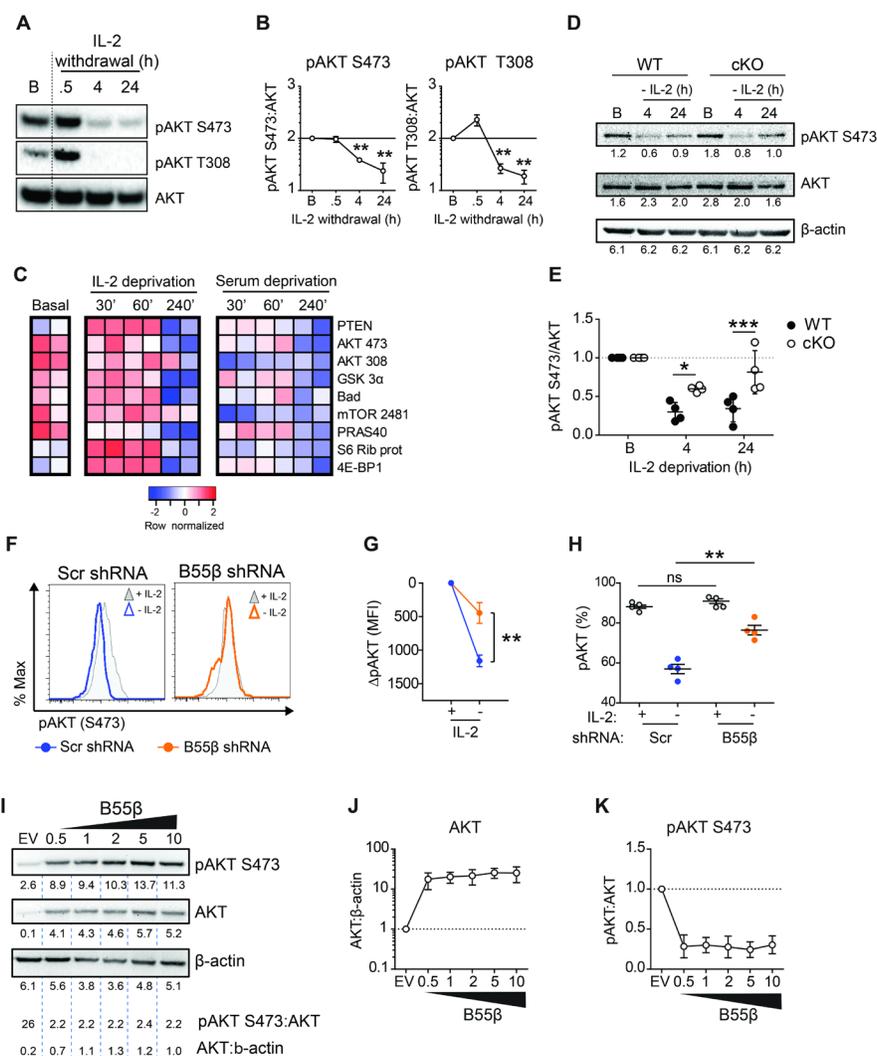


Figure 3. Cytokine withdrawal-induced AKT dephosphorylation depends on B55β. (A) Activated and expanded human T cells were resuspended in fresh RPMI (10% FBS) devoid of IL-2. AKT phosphorylation was assessed before (B) and after (0.5, 4, and 24 h) cytokine withdrawal by Western blot. A representative blot is shown ($n=5$). (B) Cumulative data of 5 experiments. Mean \pm SEM of pAKT:AKT density is shown. $**p \leq 0.01$ (paired two-tailed t test). (C) Human T cells activated and expanded were lysed at the indicated time points. Phosphorylation of the enlisted proteins was analyzed by immunoblot ($n=2$). (D) T cells from WT and cKO mice were activated and expanded. Cells were lysed at the indicated time points after IL-2 deprivation. AKT phosphorylation was quantified by Western blot. Densitometry is indicated under each blot. Two-way ANOVA with Bonferroni's multiple comparisons test $*p=0.025$; $***p=0.0006$ ($n=4$). (E) Human T cells were infected with B55β-specific or control shRNA-encoding lentiviruses. Cells were deprived of IL-2 and pAKT S473 was quantified by flow cytometry. Representative histograms: grey shades indicate pAKT prior and empty histograms pAKT after 8 hours of cytokine withdrawal. (F) Mean \pm SEM of pAKT (S473) in cells infected with control (blue circles) or B55β-specific (orange circles) lentiviruses. Mean fluorescence intensity (MFI) was normalized to Basal time point. Paired two-tailed t test; $**p \leq 0.01$. (G) The percentage of pAKT⁺ cells from (F) was quantified before (empty circles) and after IL-2 withdrawal (full circles). Each dot represents one sample. Two-tailed t test, $**p=0.001$. (H) 293 T cells were transfected with a B55β-encoding plasmid (0.5-10 μ g). Twenty-four hours later, cells were lysed and the indicated proteins were quantified by Western blotting. Densitometry is indicated. Cumulative data of 4 independent experiments showing AKT:β-actin (J) and pAKT:AKT (K) is shown.

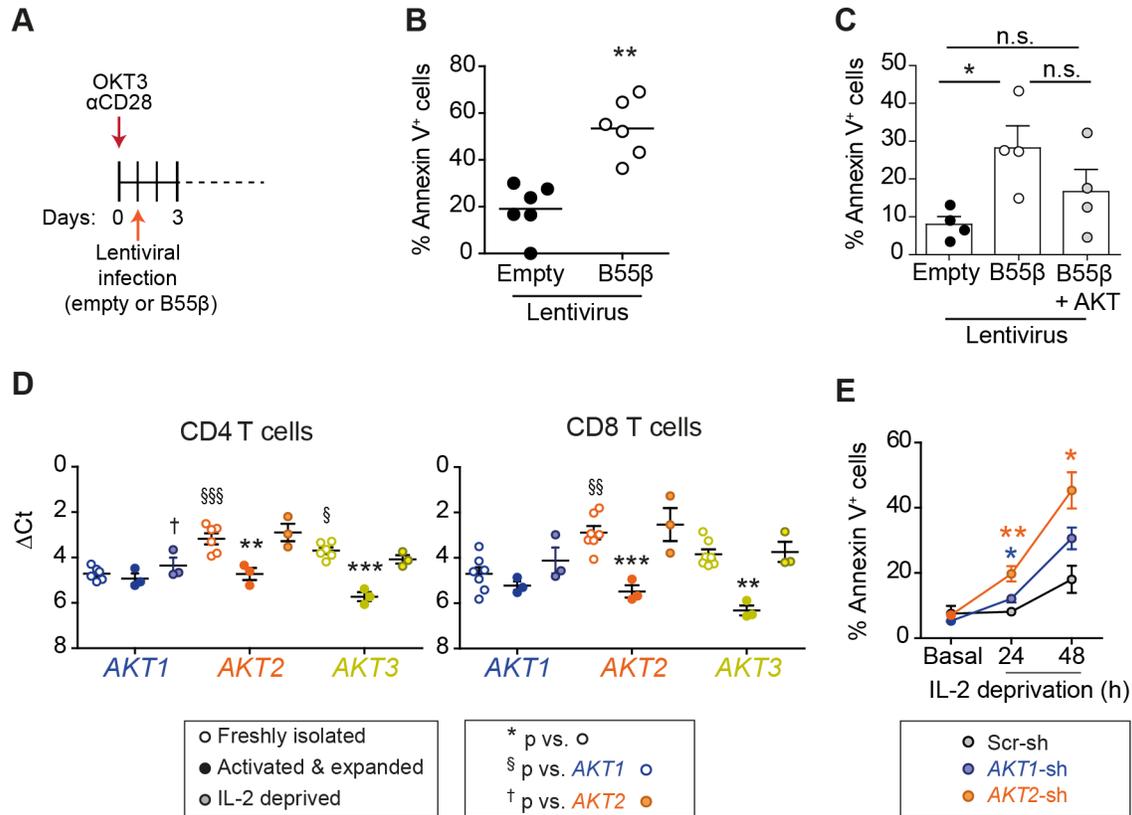


Figure 4. Apoptosis induced by B55β is blocked by AKT overexpression. (A) Schematic representation of in vitro stimulation and lentiviral infection of human T cells. (B) Apoptosis was quantified in human T cells infected with control- or B55β-encoding lentiviruses. Each dot represents a different sample (n=6); line indicates mean. Paired two-tailed t test was used; **p≤0.01. (C) Human T cells were infected with control, B55β-encoding, or B55β-encoding plus AKT-encoding lentiviruses and apoptosis was quantified by flow cytometry. Shown is the cumulative data (mean + SEM) of 4 independent experiments. One-way ANOVA with Dunn's multiple comparison test was used, *p<0.05. (D) *AKT1*, *AKT2*, and *AKT3* mRNA levels were quantified in human CD4 and CD8 T cells at the following time points: (1) immediately after isolation (empty circles); (2) after 7 days of activation (anti-CD3 + anti-CD28) and expansion in IL-2 (full circles); (3) 24 hours after IL-2 withdrawal (half-tone filled circles). Results are presented as ΔCt (vs. *BACT*). **p<0.01 and ***p<0.001 vs. same gene in freshly isolated cells; §p<0.05 and §§§p<0.001 vs. *AKT1* in freshly isolated cells; †p<0.05 vs. *AKT2* in IL-2 deprived cells. One-way ANOVA with Bonferroni's multiple comparison test was used. (E) IL-2 deprivation was induced in cells infected with control-, *AKT1*-, and *AKT2*-shRNA-encoding lentiviruses. Annexin V⁺ cells were quantified at the indicated time points. *p<0.05 and **p<0.01 vs. control (two-tailed paired Student's t test).

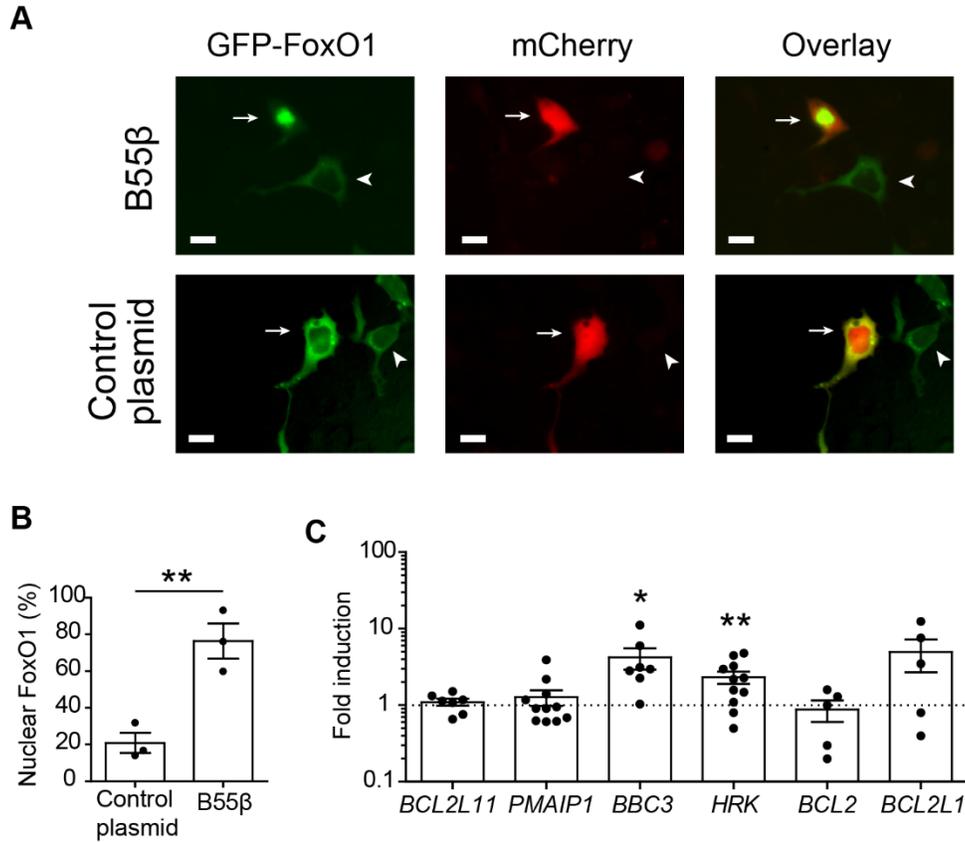


Figure 5. B55β activates FoxO1 and promotes HRK transcription. (A) MCF-7 cells were transfected with a plasmid encoding a GFP-FoxO1 fusion protein and either pLVX-PPP2R2B-IRES-mCherry or pLVX-mCherry and FoxO1 localization was evaluated by fluorescence microscopy. Shown are representative images. Arrows indicate cells transfected with both plasmids; arrowheads cells transfected only with GFP-FoxO1. Scale bar represents 5 μm. (B) Cumulative data (Mean ± SEM) of 3 independent experiments is shown (each dot represents one experiment). In each experiment, 100 cotransfected cells were evaluated. For comparison of the means, unpaired two-tailed t test was used; **p≤0.01. (C) Human T cells were infected with control- or B55β-encoding lentiviruses following the procedure depicted in Figure 4. Sixteen hours later, expression of the indicated genes was quantified by real time PCR and normalized to ACTB. Cumulative data of 8 independent experiments expressed as mean ± SEM fold induction against empty virus (indicated by the broken line). Each dot represent one sample. For comparison of the means, unpaired two-tailed t test was used; *p≤0.05, **p≤0.01.

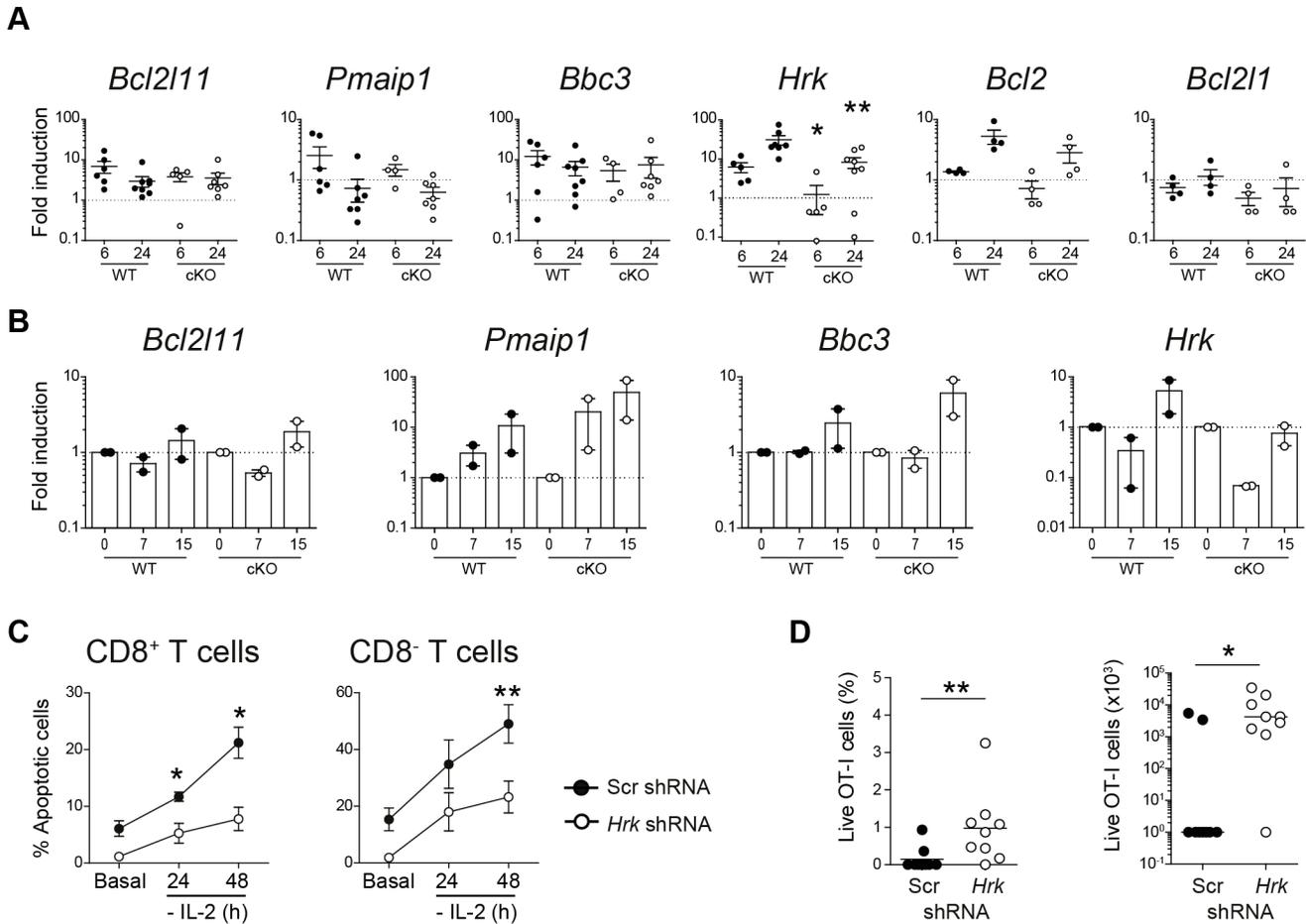


Figure 6. B55 β -induced *Hrk* is essential for CWID. (A) T cells from *Ppp2r2b*^{+/+}*Cd4*.Cre⁺ (WT) or *Ppp2r2b*^{fl/fl}*Cd4*.Cre⁺ (cKO) mice were activated and expanded in vitro. Expression of the indicated genes was assessed by qPCR before (Basal) and after 6 and 24 hours of cytokine withdrawal. Cumulative data from 4 experiments (n=3-4 mice/group/experiment) are shown. Mean and SEM are indicated. *p \leq 0.05, **p \leq 0.01 (unpaired two-tailed Mann-Whitney test). (B) WT or cKO OT-I cells were adoptively transferred into CD45.1⁺ mice. Next day, LM-OVA was injected into recipient mice. RNA was extracted from sorted cells (CD45.2⁺ CD8⁺ V α 2⁺ V β 5⁺) at days 7 and 15 and qPCR was performed. Results are presented as mean and SEM. Cumulative data from 2 independent experiments (n=3-5 mice/group/experiment) are shown. In (A) and (B), the dotted line indicates the expression level of the Basal sample. (C) T cells from WT mice were activated infected with control (Scr) or *Hrk*-shRNA encoding lentiviruses. Cells were expanded and apoptosis was induced by cytokine withdrawal. GFP⁺ (effectively infected) apoptotic cells were quantified. Cumulative data from 2 experiments (n=4 mice/group/experiment). *p \leq 0.05, **p \leq 0.01 (unpaired two-tailed t test). (D) Cytotoxic T Lymphocytes from OT-I WT or cKO mice were infected with a control (Scr-Cerulean) or an *Hrk*-specific shRNA encoding lentivirus (GFP). Six days later, 1.6x10⁴ Cerulean⁺ and GFP⁺ OT-I T cells were injected at a 1:1 ratio into RIPmOVA mice. Fourteen days later, relative and absolute numbers of transferred cells (Cerulean or GFP⁺) were quantified (n=9). *p \leq 0.05, **p \leq 0.01 (unpaired two-tailed Mann-Whitney test).

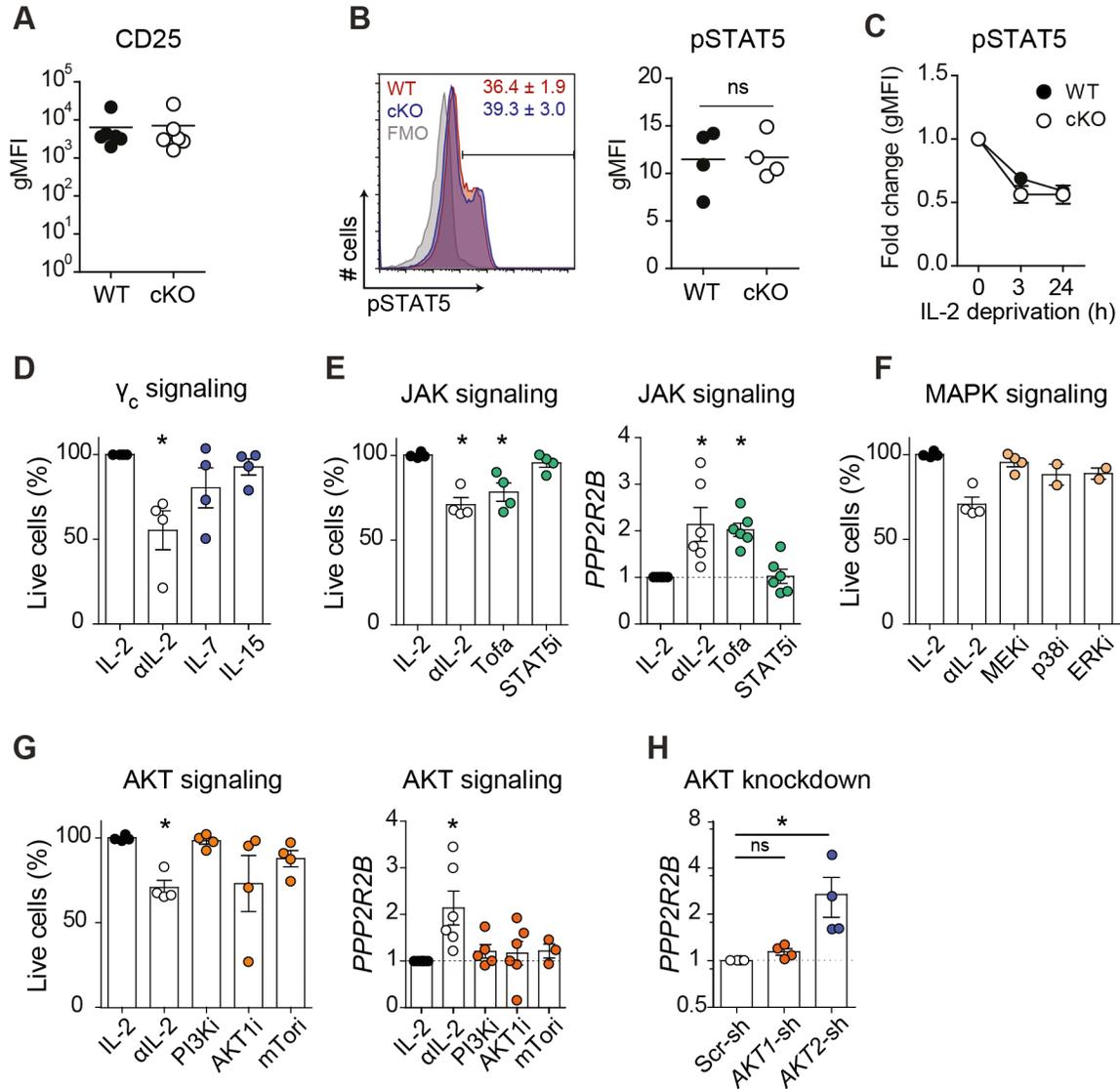


Figure 7. AKT2 inhibits B55 β expression. (A-C) *Ppp2r2b*^{+/+} (WT) or *Ppp2r2b*^{fl/fl} (cKO) T cells were activated and expanded in the presence of IL-2 for ten days. (A) CD25 (IL-2R α) expression on activated WT and cKO T cells. (B) STAT5 (Y694) phosphorylation in the presence of IL-2 (10 U/mL). A representative histogram and cumulative data from two independent experiments are shown. n.s., not significant ($p=0.46$, unpaired t test). (C) STAT5 phosphorylation in WT and cKO mice upon IL-2 withdrawal. Data are expressed as mean \pm SEM. Two independent experiments ($n=2$ per group, per experiment). (D-G) Activated and expanded T cells, were deprived of IL-2 (α IL-2) in the absence or presence of other cytokines, or were maintained in IL-2 while exposed to specific inhibitors. Live cells were quantified after 48h and compared with cells not deprived of IL-2 (IL-2). *PPP2R2B* mRNA levels were quantified 24 hours after IL-2 deprivation or after addition of inhibitors. (D) The effect of IL-7 (15 ng/mL) or IL-15 (20 ng/mL) was analyzed in cells deprived of IL-2. (E) Cells were incubated with tofacitinib (Tofa; 100 nM) or STAT5i (25 μ M). (F) Cells were exposed to PD98039 (MEKi, 10 μ M), SB202190 (p38i, 1 μ M), or FR180204 (ERKi, 20 μ M). (G) Cells were incubated with LY294002 (PI3Ki, 2 μ M) MK-2206 (AKTi, 2 μ M), or rapamycin (mTORi, 500 nM). Three independent experiments ($n=1-2$ per experiment). Kruskal-Wallis test with Dunn's multiple comparison analysis was used. * $p<0.05$. (H) T cells were activated and infected with lentiviruses encoding shRNAs (scrambled, *AKT1*-, or *AKT2*-specific). *PPP2R2B* expression was quantified by qPCR. Four independent experiments ($n=1$ per experiment). Friedman test with Dunn's multiple comparison analysis was used. * $p<0.05$.

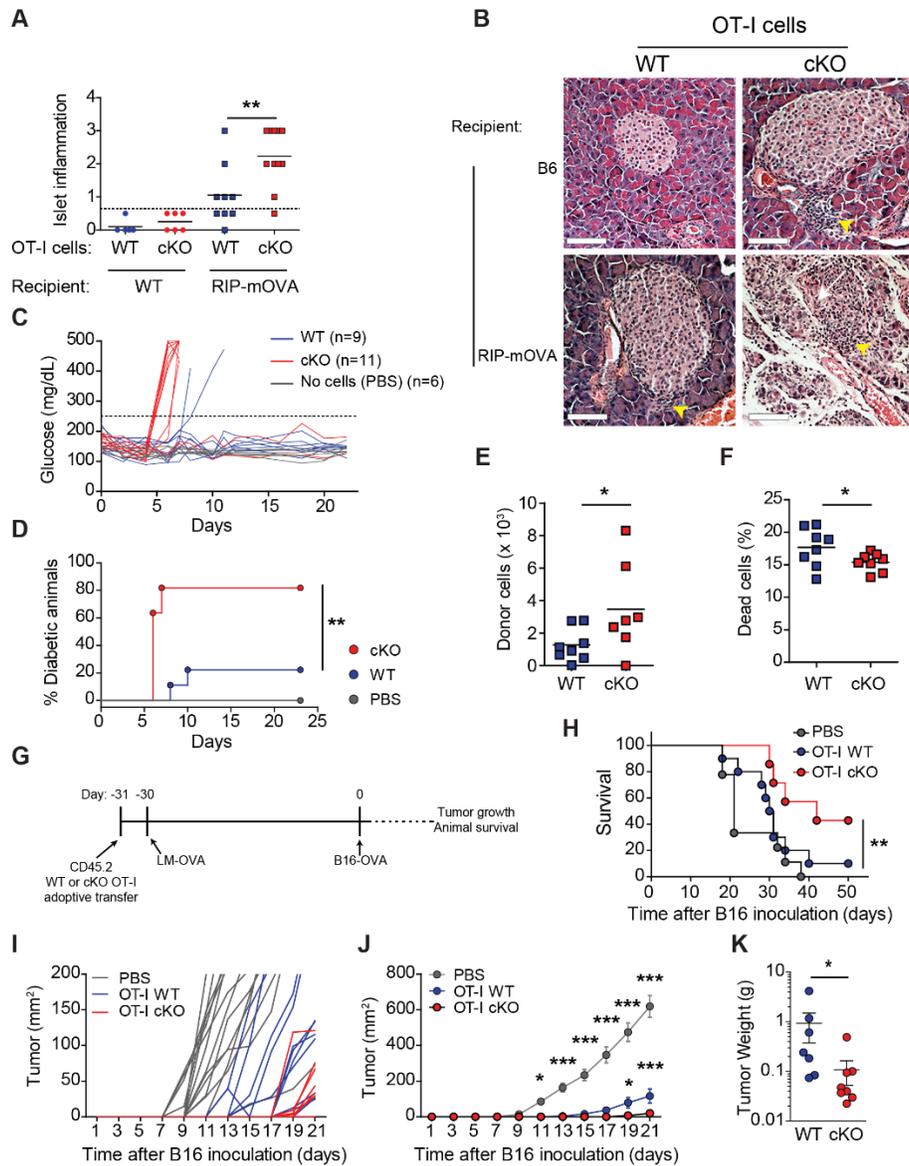


Figure 8. B55 β controls the pathogenic capacity of CD8 T cells. (A-F) OT-I cells differentiated into CTLs. After 4 days, they were i.v. injected into RIP-mOVA or WT mice. Blood glucose was quantified every day. (A) Pancreatic inflammation was scored: 0, no inflammation; 1, small perivascular infiltrates; 2, inflammatory infiltrates surrounding the islets; 3, inflammatory infiltration and/or destruction of the islets. Cumulative data of two independent experiments ($n=4-6$ mice per group) is shown. Unpaired t test, $**p \leq 0.01$. (B) Representative images of H&E stained pancreata from (A) are shown. Arrow heads indicate inflammatory infiltrates. Scale bar represents 100 μm (C) Glucose levels in RIP-mOVA mice that received WT or cKO OT-I CTL, or PBS. Cumulative data from two independent experiments is shown. (D) Incidence of diabetes in RIP-mOVA mice ($n=4-6$ mice per group). Log-rank (Mantel-Cox) test, $**p \leq 0.01$. (E) Transferred cells ($\text{CD45}^+\text{CD8}^+\text{V}\alpha 2^+\text{V}\beta 5^+\text{CD44}^+$) in draining lymph nodes and pancreata of recipient RIPmOVA mice 5 days after CTL transfer. Cumulative data of two experiments ($n=3-5$ mice per group). Unpaired t test, $*p \leq 0.05$. (F) Dead donor cells (GhostDye⁺) in the spleen of recipient mice after CTL transfer. Cumulative data two experiments ($n=3-5$ mice per group). Unpaired t test, $*p \leq 0.05$. (G-K) 10^6 OT-I CD45.2 *Ppp2r2b*^{+/+} or *Ppp2r2b*^{fl/fl} were adoptively transferred into CD45.1 mice. Next day, 10^4 c.f.u. of LM-OVA were i.v. injected into the recipient mice. After 30 days, 2×10^5 B16-OVA melanoma cell line cells were implanted in the left flank of the animals (G). Survival (H) and tumor growth was monitored. Cumulative data from two experiments with 3-5 mice/group. One-way (H) and two-way (J) ANOVA and unpaired t test (K) were used. Mean \pm SEM is depicted. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

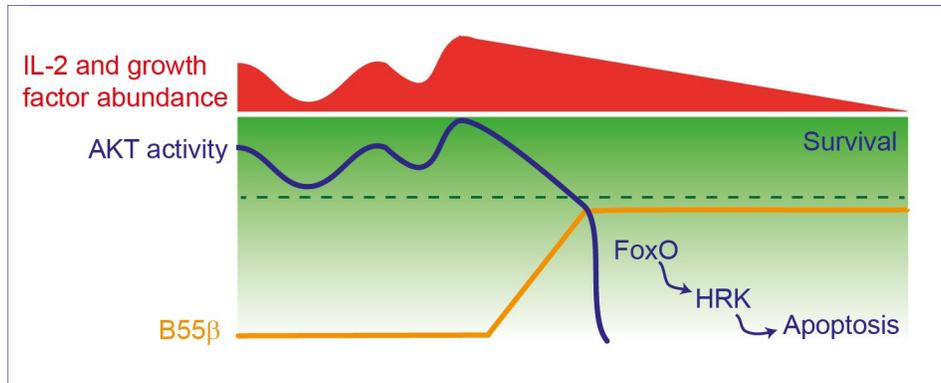


Figure 9. Decreased AKT activity induces B55 β transcription which halts further AKT activation. AKT activity fluctuates in response to nutrient and growth factor abundance. When AKT activity decreases under a certain threshold (indicated by the broken line), for example, during IL-2 withdrawal or AKT silencing, expression of B55 β is induced. By promoting AKT dephosphorylation, B55 β actively inhibits further AKT activity and commits the cell to apoptosis through the activation of FoxO and the induction of HRK.