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Mutually exclusive regulation of T cell survival by IL-7R and antigen receptor-induced signals

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Two major processes govern T cell proliferation and survival: interleukin-7-mediated homeostasis and antigen-induced selection. How cells transit between the two states is unknown. Here we show that T cell receptor ligation actively inhibits homeostatic survival signals while initiating a new, dominant survival programme. This switch is mediated by a change in the expression of pro- and anti-apoptosis proteins through the downregulation of Bcl-2 and the induction of Bim, A1 and Bcl-xL. Calcineurin inhibitors prevent the initiation of the new survival programme, while permitting the dominant repression of Bcl-2. Thus, in the presence of these drugs the response to antigen receptor ligation is cell death. Our results identify a molecular switch that can serve as an attractive target for inducing antigen-specific tolerance in treating autoimmune disease patients and transplant recipients.

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Mature T cells are subject to two regulatory processes. Homeostasis maintains the size of the peripheral pool^{1,2}, whereas antigen-receptor activation in response to pathogens leads to several rounds of proliferation, differentiation and cell death. Homeostasis of naive T cells is critically dependent on engagement of the interleukin (IL)-7 receptor (IL-7R)³ together with a weak, tonic T cell receptor (TCR) stimulus provided by major histocompatibility complex (MHC) proteins loaded with self peptides^{4–7}. T cells removed from their homeostatic environment and placed into culture die rapidly. This T cell death can be prevented by addition of IL-7 to the culture medium⁸. In contrast, agonistic antibodies or antigenic peptides presented by MHC proteins initiate strong signalling via the TCR, and the resulting responses are enhanced in a quantitative manner by costimuli, such as those provided by CD28 and cytokines (for example, IL-2)^{9–12}.

The tightly regulated expression of pro- and anti-apoptotic molecules is essential to control T cell survival and death during steady state, TCR repertoire selection and TCR activation-driven proliferation of foreign antigen-specific T cells. The pro- and anti-apoptotic members of the Bcl-2 protein family have critical roles in T cell survival throughout differentiation. IL-7-/IL-7R-mediated survival is dependent on the anti-apoptotic Bcl-2, (refs 13,14) Mcl-1 (refs 15,16) and the inhibition of pro-apoptotic Bim^{17–19}. Conversely, during antigenic stimulation of T cells via their TCR, their survival is controlled by the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-xL, Mcl-1 and A1 (refs 15,20,21), and the pro-apoptotic Bim^{22,23}.

Although the need for careful regulation of survival during both homeostasis and immune activation is well described, the mechanisms that control the transition between the two states, and how signalling conflicts could be avoided are not known.

Here we show that TCR stimulation initiates a new, dominant survival programme, while simultaneously switching off IL-7- and Bcl-2-mediated homeostatic survival. Furthermore, calcineurin and MEK inhibitors prevent TCR-induced expression of new pro-survival proteins, while leaving intact the inhibition of homeostatic survival. As a consequence, these drugs facilitate TCR-induced cell death, revealing a potential method for therapeutic tolerance induction.

Results

TCR ligation inhibits IL-7-/IL-7R-mediated T cell survival.

Naive T cells rapidly undergo apoptosis in culture with a half-life of 1–2 days^{8–10} (Fig. 1a). Mitogenic stimulation with agonistic antibodies to the TCR/CD3 complex does not affect this cell loss over the first 24 h, resulting in only a proportion of cells reaching the first cell division^{9,10}. As the homeostatic regulator IL-7 substantially inhibits the death of naive, unstimulated T cells in culture⁸ (Fig. 1a), we were surprised that IL-7 had no additive effect on anti-CD3-induced T cell proliferation (Fig. 1a). To explore this further, we examined T cell survival in culture at time points shortly after TCR stimulation (Fig. 1b). Addition of IL-7 enhanced survival of unstimulated but not TCR-stimulated T cells, indicating that TCR ligation actively inhibited IL-7-/IL-7R-mediated survival signalling. This inhibitory effect was seen in both CD4⁺ (Fig. 1b) and CD8⁺ T cells (Fig. 1c).

T cells from TCR transgenic (tg) mice were tested to examine whether the inhibition of IL-7-/IL-7R-mediated survival (Fig. 1a–c) also occurred with cognate MHC/peptide stimulation. CD8⁺ T cells from OT-I TCR tg or CD4⁺ T cells from OT-II and DO11.10 TCR tg mice were stimulated with SIINFEKL peptide or with antigen-presenting cells (APCs) pulsed with OVA_{323–339},

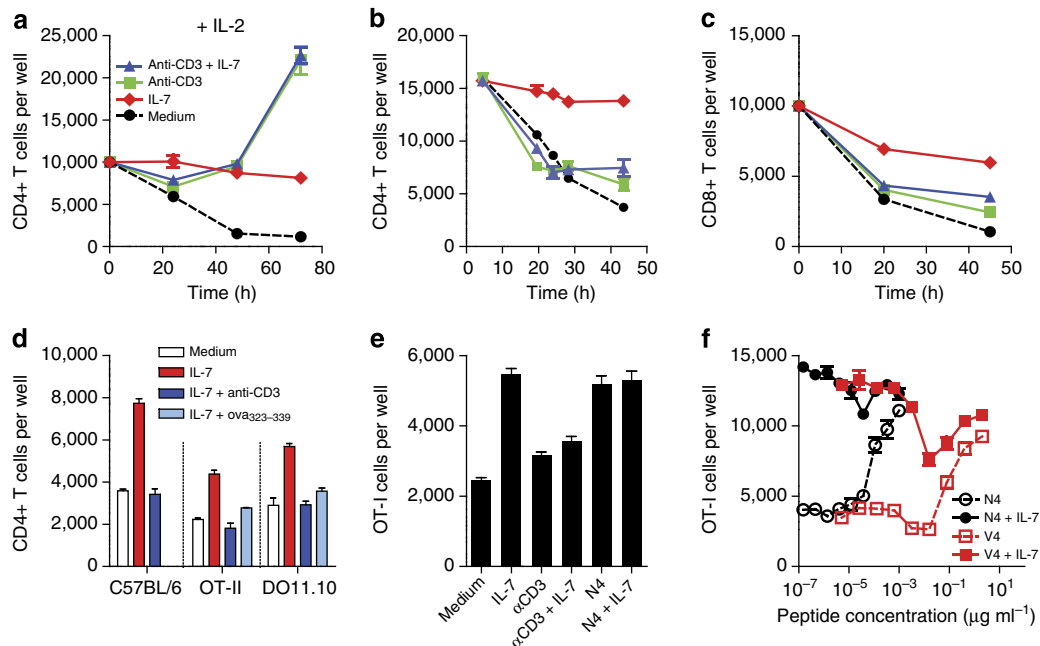


Figure 1 | TCR stimulation inhibits IL-7-mediated survival. Purified CD4⁺ or CD8⁺ T cells were stimulated *in vitro* and total viable cells were measured at time points indicated. **(a, b)** Time course of viable CD4⁺ T cells from C57BL/6 mice cultured in medium alone or after stimulation with plate-bound anti-CD3 with and without 1 ng ml⁻¹ IL-7 in the presence of 100 Ug ml⁻¹ IL-2 **(a)** or without IL-2 **(b)**. **(c)** Time course of viable CD8⁺ T cells from C57BL/6 mice cultured in medium alone or after stimulation with plate-bound anti-CD3 with and without 1 ng ml⁻¹ IL-7. **(d)** CD4⁺ T cells from C57BL/6, OT-II and DO11.10 TCR tg mice were cultured for 16 h in medium with or without 1 ng ml⁻¹ IL-7, or stimulated with plate-bound anti-CD3 or OVA peptide, and autologous splenocytes in the presence of 1 ng ml⁻¹ IL-7. **(e)** OT-I CD8⁺ T cells were cultured for 22 h in medium or stimulated with plate-bound anti-CD3 or 0.01 μg ml⁻¹ N4 peptide with and without 1 ng ml⁻¹ IL-7. **(f)** CD8⁺ OT-I cells were stimulated for 20 h with a range of N4 (10⁻⁷–10⁻³ μg ml⁻¹) or V4 (5 × 10⁻⁶–2 μg ml⁻¹) peptide concentrations in medium with or without 1 ng ml⁻¹ IL-7. Symbols and colours of **b** and **c** as in **a**. Data are presented as means ± s.e.m. of triplicate cultures.

respectively, and cell viability was measured 20 h after stimulation, just before cells entering their first division²⁴. As seen with CD3 antibodies, TCR stimulation with peptide-pulsed APCs inhibited IL-7-/IL-7R-mediated survival in CD4⁺ T cells (Fig. 1d). However, no cell loss was observed after stimulation of OT-I CD8⁺ T cells with SIINFEKL peptide (N4; Fig. 1e). As stimulation of OT-I T cells with this high-affinity peptide enhances cell survival²⁴ (Fig. 1e), we hypothesized that activation of a different survival programme was masking the inhibition of the IL-7-/IL-7R-mediated survival in this setting. As the OT-I TCR has a higher affinity for its cognate peptide-MHC compared with the OT-II TCR²⁵, we also speculated that the affinity of the TCR/MHC-peptide interaction may affect the regulation of these two cell survival programmes. To test these hypotheses, we stimulated OT-I T cells with a number of peptide variants with different affinities (amino acid replacements at position four or seven of the N4 peptide SIINFEKL) in the absence or presence of IL-7 and measured their survival after 20 h. In the absence of IL-7, increasing concentrations of each peptide led to enhanced T cell survival (Fig. 1f and Supplementary Fig. S1). In the presence of IL-7, stimulation with peptide concentrations at the lower range of efficacy resulted

in a reduction in T cell survival (Fig. 1f and Supplementary Fig. S1). This reduction is consistent with a transition point from one survival programme to another (identified by arrows in Supplementary Fig. S1). These results reveal a complex net effect of TCR stimulation on T cell survival, where IL-7-/IL-7R-mediated survival signalling was inhibited, while a new survival programme was induced, the potency of the latter survival programme correlating with the affinity of the TCR stimulation.

Evidence for two mechanisms of inhibition by TCR ligation. As pSTAT5 has been described as a target for impaired γ c-cytokine signalling through persistent TCR engagement²⁶ and as IL-7-/IL-7R-mediated cell survival depends on STAT5 (refs 27,28) to directly drive *Bcl-2* expression^{13,14}, we investigated the effect of TCR ligation on both STAT5 phosphorylation and *Bcl-2* mRNA expression levels. STAT5 was rapidly phosphorylated in T cells after exposure to IL-7, and it only marginally declined over 6 h as measured by flow cytometry (Fig. 2a). A progressive loss of pSTAT5 was observed within 2 h of TCR stimulation in T cells that had been pre-exposed to IL-7 (Fig. 2a), whereas total STAT5 remained relatively constant (Supplementary Fig. S2). This early

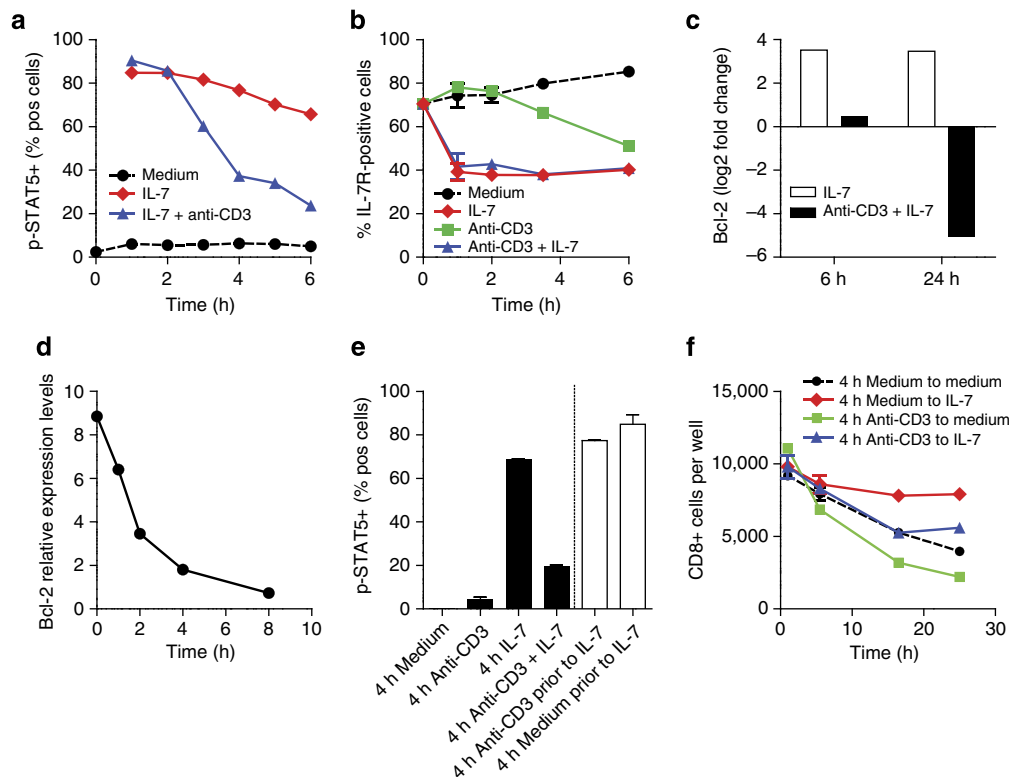


Figure 2 | TCR ligation inhibits *Bcl-2* and phosphorylation of STAT5. (a) Time course of percentage of cells positive for phospho-STAT-5 of CD4⁺ T cells from C57BL/6 mice cultured in medium alone, with 10 ng ml⁻¹ IL-7 or stimulated with plate-bound anti-CD3 with 10 ng ml⁻¹ IL-7 measured by flow cytometry. (b) Expression of IL-7R α on CD8⁺ T cells from C57BL/6 mice cultured in medium or stimulated with plate-bound anti-CD3 with or without 1 ng ml⁻¹ IL-7. IL-7R α levels were measured by flow cytometry at indicated times and expressed as percentage IL-7R α -positive cells. (c) Relative expression levels of *bcl-2* mRNA in CD4⁺ T cells from C57BL/6 mice cultured in 1 ng ml⁻¹ IL-7 alone or stimulated with plate-bound anti-CD3 + 1 ng ml⁻¹ IL-7 measured by quantitative PCR (qPCR). Data are expressed as log₂ of the fold change: *bcl-2* mRNA expression relative to *bcl-2* levels in freshly isolated cells. (d) Relative expression levels of *bcl-2* mRNA of CD4⁺ from C57BL/6 mice T cells stimulated with anti-CD3 after overnight culture in IL-7 measured by qPCR. Data are expressed as expression relative to freshly isolated cells. (e) Loss of STAT5 phosphorylation is reversible after removal from antigen stimulus. Phosphorylation of STAT5 was measured by flow cytometry in CD8⁺ T cells unstimulated or stimulated with plate-bound anti-CD3 before exposure to IL-7. CD8⁺ T cells from C57BL/6 mice were cultured either continuously for 4 h in medium or on CD3 antibody-coated plates with or without 1 ng ml⁻¹ IL-7 (filled columns) or were cultured in medium or on CD3 antibody-coated plates for 4 h without IL-7 before being removed from stimulus, and then exposed to 1 ng ml⁻¹ IL-7 for 15 min (open columns). (f) Removal from TCR stimulation does not fully restore IL-7-mediated survival. CD8⁺ T cells C57BL/6 mice were cultured in medium or on anti-CD3-coated plates for 4 h before being removed from stimulus (indicated as 0 h), and cultured in new medium with or without 1 ng ml⁻¹ IL-7 for up to 24 h. Data represent viable cells per well. (b, e, f) Data are presented as means \pm s.e.m. of triplicate cultures.

inhibition was not due to an effect on IL-7R expression, as TCR stimulation had no impact on the rapid downregulation of IL-7R induced by IL-7 signalling in CD4⁺ (Supplementary Fig. S2) and in CD8⁺ T cells (Fig. 2b). Furthermore, Tyr1022/1023 phosphorylation of Jak-1, the kinase that links IL-7R ligation to STAT5 activation, was not affected by TCR stimulation (Supplementary Fig. S2). As Jak-STAT signalling is negatively regulated by the suppressor of cytokine signalling (SOCS) proteins²⁹, we examined the effect of loss of SOCS1, -2 or -3 on the TCR stimulation-induced inhibition of IL-7-/IL-7R-mediated T cell survival. Deficiency in these SOCS proteins did not rescue IL-7-/IL-7R-mediated T cell survival after TCR stimulation, indicating that these SOCS proteins are not involved in this process (Supplementary Fig. S3).

Consistent with the loss of pSTAT5, expression of *Bcl-2* mRNA by IL-7 was potentially antagonized by TCR stimulation (Fig. 2c). Furthermore, pre-exposure to IL-7 followed by TCR stimulation resulted in a rapid loss of *Bcl-2* mRNA (Fig. 2d), confirming the active inhibitory cross-talk between the TCR and the IL-7R signalling pathways. However, despite the loss of mRNA, *Bcl-2* protein does not diminish before 24 h (data shown below in Fig. 4d), indicating that inhibition of the IL-7 signalling pathway leading to reduction in *bcl-2* mRNA expression is not sufficient to explain the rapid effect of TCR ligation on IL-7-mediated survival. Further evidence for a second inhibitory mechanism was provided by studies showing that loss of STAT5 phosphorylation following TCR ligation was rapidly reversible. When T cells were stimulated for 4 h with anti-CD3, and then removed from stimulus before exposure to IL-7, STAT5 phosphorylation was fully restored (Fig. 2e). However, despite the restoration of pSTAT5 levels, cell survival did not fully recover and remained markedly inhibited (Fig. 2f), revealing an additional, slower-to-reverse mechanism for inhibition of T cell survival.

Two distinct effects of TCR stimulation on survival. We sought a tool for distinguishing the survival programmes induced by IL-7/IL-7R and TCR stimulation, respectively. The calcineurin inhibitor cyclosporine A (CsA) is a potent inhibitor of some, but not all, signals emanating from TCR ligation^{30,31}. We examined the impact of CsA on OT-I T cells stimulated with various concentrations of the high-affinity N4 peptide in the absence or presence of IL-7 (Fig. 3a). As shown above (Fig. 1f), T cells cultured in medium without IL-7 showed enhanced survival with increasing peptide concentration. Addition of CsA resulted in a pronounced reduction in T cell survival as the peptide concentration was increased. A substantial inhibition of T cell survival was also noted in the presence of peptide plus IL-7. Moreover, CsA had no impact on the survival of unstimulated (no peptide) T cells, cultured in the presence or absence of IL-7 (Fig. 3a). Similar results were obtained when OT-I T cells were stimulated with the low-affinity peptide V4 (Fig. 3b). Although some loss of T cells was observed even at high V4 concentrations in the presence of IL-7, the loss of T cells in the presence of CsA was reduced compared with stimulation with the high-affinity N4 peptide. Thus, the affinity of TCR ligation affects the potency of inhibition of IL-7-/IL-7R-mediated T cell survival. FK506 (Tacrolimus), which also inhibits the calcineurin pathway, had similar effects as CsA (Supplementary Fig S4).

Pharmacological inhibitors were also sought to inform the signalling pathway that antagonizes IL-7-mediated survival. Inhibitors of protein kinase C³², phosphatidylinositol 3-kinases³³ and MEK1/2 (ref. 34) all failed to prevent TCR stimulation-mediated inhibition of IL-7-/IL-7R-driven T cell survival (Supplementary Fig. S4) ruling out a role for these

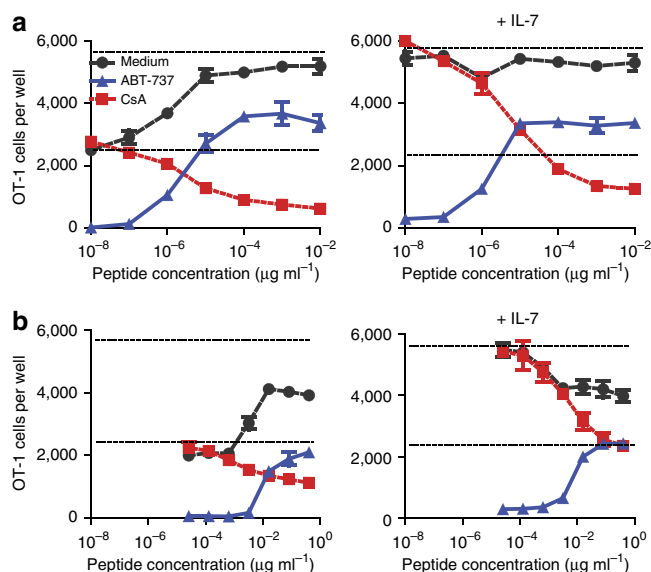


Figure 3 | A switch in survival regulation after TCR stimulation. Effects of CsA and ABT-737 on the survival of OT-1 CD8⁺ after antigen stimulation were measured. OT-1 CD8⁺ were stimulated for 22 h with (a) N4 (10^{-8} – 10^{-2} $\mu\text{g ml}^{-1}$) or (b) V4 (2.6×10^{-5} to 4×10^{-1} $\mu\text{g ml}^{-1}$) in the presence of $1 \mu\text{g ml}^{-1}$ CsA or $10 \mu\text{M}$ ABT-737 in medium (left panels) or in 1 ng ml^{-1} IL-7 (right panels). CsA was added at the start of culture, whereas ABT-737 was added 6 h after beginning of culture. Data show number of viable cells per well. Horizontal dotted lines represent number of live cells without stimulation in medium (lower line) or 1 ng ml^{-1} IL-7 (upper line). Data are presented as means \pm s.e.m. of triplicate cultures.

pathways. Notably, however, the MEK1/2 inhibitor U0126 enhanced T cell death in the presence of IL-7 in a manner similar to CsA (Supplementary Fig S4), identifying a further role for MEK/ERK signalling in promoting the new survival programme of T cells following TCR ligation.

Our results showing T cell survival could improve despite active inhibition of *Bcl-2* mRNA following TCR stimulation implied that survival after activation was not mediated by this molecule. To further elucidate the role of *Bcl-2* after T cell activation, we used the BH3 mimetic drug ABT-737 (ref 35), for which *Bcl-2* has been shown to be the main target in lymphoid cells *in vivo*³⁶. Although ABT-737 readily killed OT-I T cells stimulated with very low concentrations of N4 or V4 peptides, with increasing peptide concentrations ABT-737 killed only a relatively minor fraction of OT-I T cells (Fig. 3a, b). This effect was independent of the presence of IL-7 (Fig. 3a, b). Thus, TCR stimulation-induced T cell survival is relatively insensitive to ABT-737, indicating that *Bcl-2* (and possibly also *Bcl-xL* and *Bcl-w*) has only a limited role in this process, and that other anti-apoptotic proteins must be induced by TCR activation. Taken together, these data show that IL-7/IL-7R and TCR stimulation promote T cell survival through distinct signalling pathways and downstream molecular components. Furthermore, they confirm that the TCR-induced programme is dominant and inhibitory over IL-7-/IL-7R-mediated homeostatic T cell survival.

TCR ligation induces pro- and anti-apoptotic *Bcl-2* molecules. Various members of the *Bcl-2* family have been shown to have critical roles in the regulation of T cell survival after TCR stimulation^{15,20,21,37}. To better understand the regulation of the pro-survival members of the *Bcl-2* family in the transition from IL-7-/IL-7R- to TCR-driven cell survival, we measured their

mRNA levels over time. CD4⁺ T cells exposed to IL-7 expressed high levels of *bcl-2* and detectable *mcl-1* (Fig. 4a, top). As shown above (Fig. 2c, d) after TCR signalling *bcl-2* mRNA was distinctly downregulated within 2 h. In contrast, *A1* and *bcl-xL* mRNA levels started to increase over the same time frame (Fig. 4a, top). By 6 h, *A1* mRNA levels had increased 33-fold and *Bcl-xL* 18-fold. The induction of these proteins after TCR stimulation was also evident on protein level as measured by western blot (Fig. 4a, bottom). As treatment with CsA increases the death of T cells that had been stimulated through their TCR, we investigated the effect of this drug on the expression of these anti-apoptotic Bcl-2 family members. As previously shown²¹, CsA inhibited the TCR stimulation-induced increase in *A1* and *bcl-xL* mRNA levels (Fig. 4b). In contrast, the downregulation of *bcl-2* mRNA was not affected by this drug (Fig. 4b). These observations are consistent with our proposed switch in survival modalities between IL-7/IL-7R versus TCR signalling. Additional analysis revealed that inhibition of MEK1/2 also diminished TCR stimulation-induced upregulation of *A1* and *bcl-xL* mRNA, but did not affect the downregulation of *bcl-2* mRNA levels (Supplementary Fig. S4). These results indicate that calcineurin and MEK1/2 signalling is critical for the TCR stimulation-activated T cell survival programme, but do not affect its blockade of the IL-7-/IL-7R-mediated survival pathway.

To examine their dependence on TCR signal strength for the switch between the two distinct T cell survival programmes

(IL-7-/IL-7R- versus TCR-driven) correlates with the regulation of the expression of the different anti-apoptotic Bcl-2 family members, we stimulated OT-1 CD8⁺ T cells with increasing concentrations of N4 peptide. After TCR stimulation, the transition from *bcl-2* to *A1* expression was fastest after strong stimulation with a high dose of N4 peptide with the speed of transition in expression of these genes correlating with both the concentration (Fig. 4c) and the affinity (data not shown) of the stimulatory peptides used.

On the basis of the data shown in Fig. 2a–f, we hypothesized that the active process leading to the death of a proportion of T cells after TCR stimulation (both in the presence or absence of IL-7) might be mediated through induction of pro-apoptotic members of the Bcl-2 family. To test this hypothesis, we examined the expression of pro-apoptotic Bcl-2 family members in T cells before and after TCR ligation. The BH3-only protein BimL and BimEL isoforms, which are interchangeable³⁸, were strongly induced by exposure to anti-CD3, independently of the presence of IL-7 (Fig. 4d). A substantial increase in the multi-BH domain pro-apoptotic Bcl-2 family member Bax was also seen under these conditions (Fig. 4d). As noted earlier, relatively little change in Bcl-2 protein levels was observed up to 24 h, which is presumably explained by the long half-life (~20 h) of this protein³⁹. Pro-apoptotic activity of Bax is inhibited by binding of anti-apoptotic Bcl-2 family proteins, including Bcl-2 to this molecule. On the other hand,

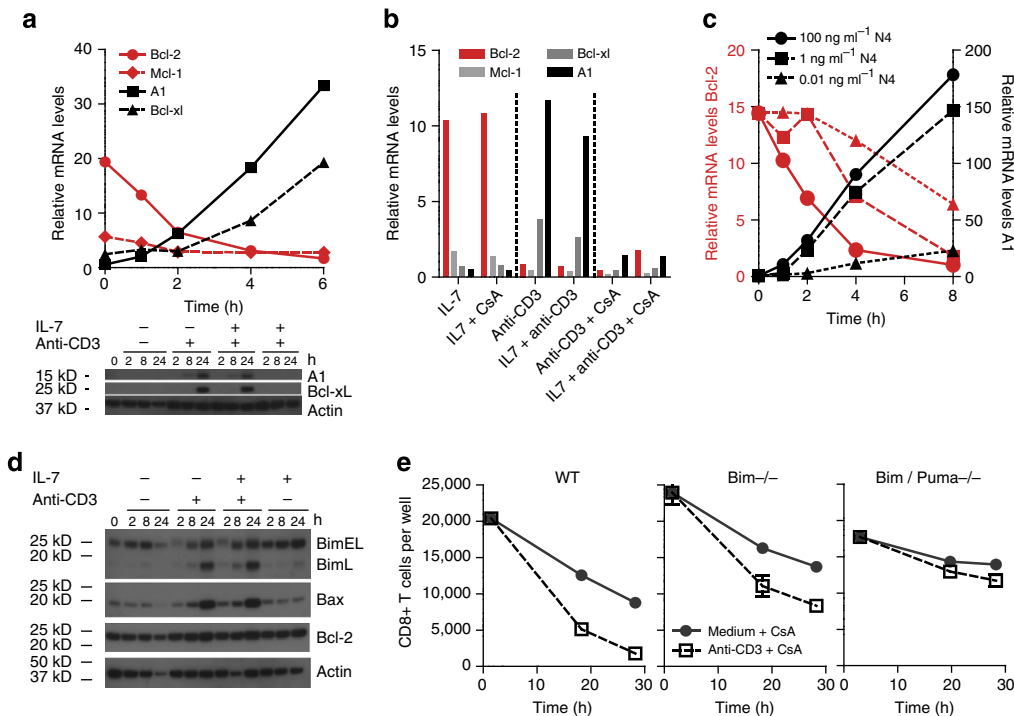


Figure 4 | TCR signalling alters Bcl-2 family gene expression. (a) Relative expression levels of *bcl-2*, *mcl-1*, *A1* and *bcl-xL* mRNA measured by quantitative PCR (qPCR) in CD4⁺ T cells from C57BL/6 mice cultured overnight with 10 ng ml⁻¹ IL-7 before stimulation with plate-bound anti-CD3. Time represents time after exposure to anti-CD3 (top). Protein expression of A1 and Bcl-xL (with actin as the loading control) in CD8⁺ T cells from C57BL/6 mice cultured for the time point indicated in medium or on CD3 antibody-coated plates with or without 10 ng ml⁻¹ IL-7 (bottom). (b) qPCR analysis of *bcl-2*, *mcl-1*, *A1* and *bcl-xL* mRNA in CD4⁺ T cells from C57BL/6 mice cultured overnight with 10 ng ml⁻¹ IL-7 before culture for 6 h in medium or on plate-bound CD3 antibodies in presence or absence of CsA (2.5 μg ml⁻¹) and presence or absence of 10 ng ml⁻¹ IL-7. (c) Time course of relative expression levels of *bcl-2* and *A1* mRNA in OT-1 CD8⁺ T cells pre-cultured with IL-7 overnight before stimulation with cognate N4 peptide (0.01, 10 or 100 ng ml⁻¹) measured by qPCR. Time represents time after exposure to N4 peptide. (a–c) qPCR data are expressed as relative mRNA expression relative to freshly isolated uncultured cells. (d) Western blot analysis of Bim, Bax and Bcl-2 (with actin as the loading control) in CD8⁺ T cells from C57BL/6 mice cultured in medium or on CD3 antibody-coated plates with or without 10 ng ml⁻¹ IL-7. (e) Effect of loss of Bim and Puma on survival of CD8⁺ T cells after TCR ligation. CD8⁺ T cells from C57BL/6-wild type (WT), Bim^{-/-} or Bim/Puma^{-/-} were cultured in medium or on CD3 antibody-coated plates in the presence of 1 μg ml⁻¹ CsA. Total viable cells were measured at indicated time points. Data are presented as means ± s.e.m. of triplicate cultures.

Bim binds to Bcl-2, which in turn releases Bax⁴⁰. Hence, induction of either Bax or Bim counteract Bcl-2 and (particularly together) explain that cells can die upon induction of these molecules even without an apparent loss of Bcl-2.

Gene-targeted mice were used to assess the function of Bim and other pro-apoptotic BH3-only proteins in the TCR stimulation-induced killing of T cells. We found the death of T cells induced by stimulation with anti-CD3 in the presence of CsA was partially inhibited by loss of Bim (Fig. 4e). Although loss of Puma had a small but noticeable effect (Supplementary Fig. S5), the combined loss of both Bim and Puma afforded even greater protection (Fig. 4e). In contrast, loss of Noxa, Bid or Bad had no impact on the survival of T cells under these conditions (Supplementary Fig. S5). Similar results were found when IL-7 was included in culture (Supplementary Fig. S5). Thus, the induction of pro-apoptotic BH3-only protein Bim, working in concert with Puma, has a critical role in TCR stimulation-induced blockade of IL-7-dependent, Bcl-2-mediated, T cell survival.

IL-2 promotes survival independently of STAT5. Addition of exogenous IL-2 is reported to partially overcome the inhibitory effects of CsA on TCR stimulation-induced T cell proliferation^{41,42}. In contrast to IL-7, which had no impact on T cell survival following TCR stimulation, addition of exogenous IL-2 improved survival before cells entered their first division (Fig. 5a). The additional survival provided by IL-2 is sufficient to allow extra cells to undergo division in the presence of CsA, resulting in a significant overall response (Fig. 5b). This result is slightly paradoxical as IL-2, similar to IL-7, can signal through the STAT5 pathway. However, the increased survival seen with IL-2 was mediated without altering STAT5 phosphorylation/activation levels, indicating an alternative pathway is being used (Fig. 5c).

Selective loss of antigen-specific T cells *in vivo*. The *in vitro* data taken together show that T cell activation with antigen leads to active inhibition of IL-7-/IL-7R-driven survival, and that CsA suppresses the induction of the TCR-induced (replacement) survival programme (summarized in Fig. 6). We sought evidence for the operation of these processes *in vivo* by exposing adoptively transferred OT-1 cells to antigen in the presence of the calcineurin inhibitor FK506 (Fig. 7). In a similar manner to the *in vitro* observation, a rapid loss of OT-1 T cells transferred into wild-type C57BL/6 mice was observed after *in vivo* activation with N4 peptide and before the OT-1 T cells entered the first

division (Fig. 7a, b). This loss was even greater when mice were treated with FK506 (Fig. 7a, b and Supplementary Table S1). Killing was specific to the antigen-specific OT-1 cells, as the host (C57BL/6) TCR V α 2⁺ CD8⁺ T cell population was not affected (Supplementary Table S2). Although not all OT-1 T cells were deleted through antigen stimulation in the presence of FK506, proliferation of OT-1 T cells in response to N4 peptide was reduced fivefold at 43 h when peptide stimulation occurred in the presence of FK506 compared with the peptide stimulation alone (Fig. 7b), and these cells were less progressed through division rounds (Fig. 7c). This incomplete blockade of survival and division of peptide-stimulated T cells may indicate that the efficacy of

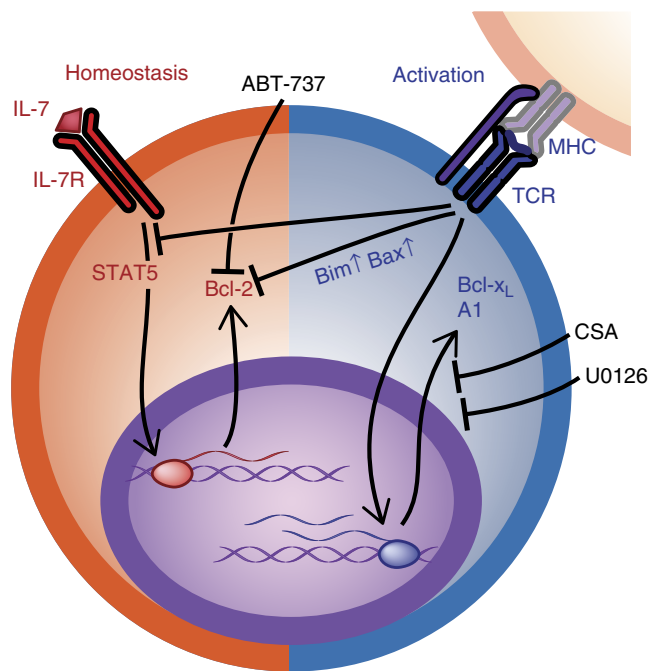


Figure 6 | Summary of interactions of TCR and IL-7R signals and inhibitory drugs. IL-7 mediates cell survival through induction of Bcl-2 (red section). TCR signalling inhibits *bcl-2* while upregulating pro-survival molecules *bcl-xL* and *A1* at the same time as pro-apoptotic proteins Bim and Bax are increased (blue). Inhibition of calcineurin or MEK1/2 with CsA or U0126, respectively, blocks TCR-mediated *A1* and *bcl-xL* induction, but has no effect on inhibition of *bcl-2*.

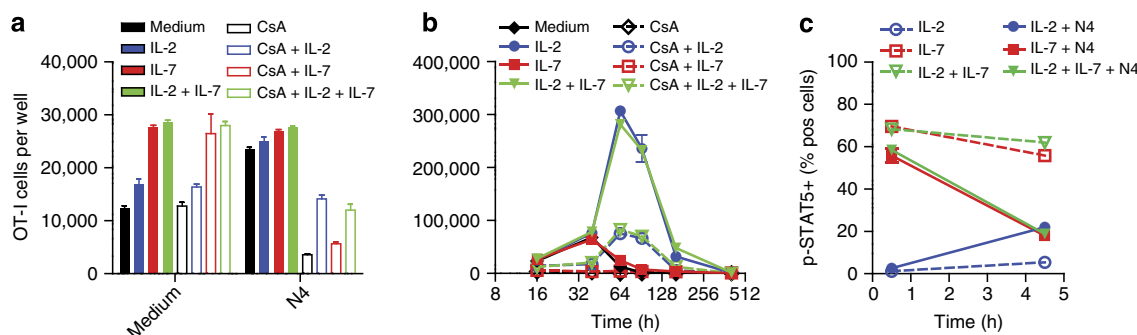


Figure 5 | IL-2 enhances survival but does not affect inhibition of STAT5 phosphorylation. OT-1 CD8⁺ T cells were cultured in medium or stimulated with 0.01 $\mu\text{g ml}^{-1}$ N4 peptide in the presence or absence of 100 U ml^{-1} IL-2 and 1 ng ml^{-1} IL-7. (a, b) IL-2 partially overcomes CSA-mediated inhibition on TCR stimulation. Number of total viable OT-1 CD8⁺ was measured after culture in the presence or absence of cytokines and with or without 1 $\mu\text{g ml}^{-1}$ CsA 16 h post stimulation (a) or over a time course up to 400 h post stimulation (b). (c) IL-2 does not affect stimulation-induced inhibition of pSTAT5. Phosphorylation of STAT5 in OT-1 CD8⁺ T cells was measured by flow cytometry after incubation for 30 min and 4.5 h with medium or N4 peptide in the presence of IL-2 or IL-7. (a-c) Data are presented as means \pm s.e.m. of triplicate cultures.

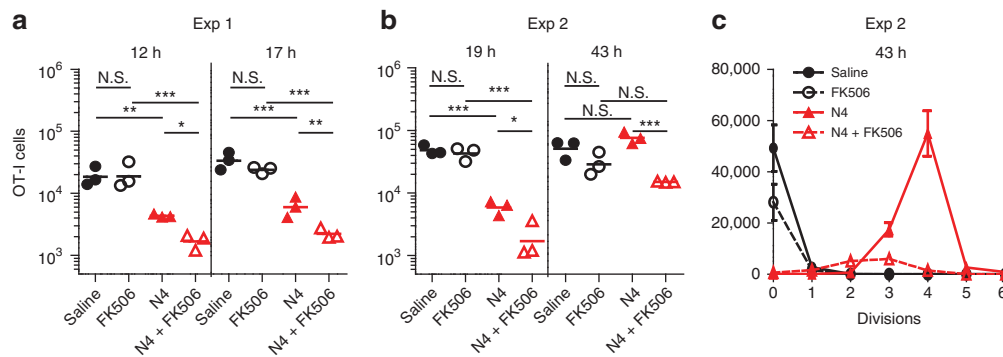


Figure 7 | FK506 enhances loss of antigen-specific T cells after TCR stimulation *in vivo*. CTV-labelled OT-I CD8⁺ T cells were adoptively transferred into wild-type C57BL/6 mice. Commencing the following day, mice were treated with 2.5 mg kg⁻¹ FK506 or saline (control groups) every 6 h until the end of the experiment. Peptide-stimulated groups received 10 μg N4 1 h (a) or 2 h (b, c) after FK506 treatment had begun. (a) Total number of OT-I TCR-tg CD8⁺ T cells recovered from pooled spleen and lymph node (LN) at 12 or 17 h post immunization. (b) Total number of OT-I TCR-tg CD8⁺ T cells recovered from pooled spleen and LN at 19 and 43 h post immunization. Geometric means of data points are shown with results of Tukey multiple comparison test from analysis of variance *0.01 < P < 0.05; **0.001 < P < 0.01; ***P < 0.001; N.S., not significant. (c) Number of OT-I TCR-tg CD8⁺ T cells per division recovered from spleen and LN 43 h post immunization as determined by CTV dilution. Data are presented as means ± s.e.m., n = 3.

FK506 is diminished *in vivo* (compared with *in vitro*), or that FK506-insensitive signals can promote survival of antigen-stimulated T cells *in vivo*, as seen with the addition of IL-2 *in vitro* (Fig. 5a).

Discussion

Here we report that T cell survival is reprogrammed upon TCR-mediated activation by both an active inhibition of sensitivity to homeostatic IL-7/IL-7R signals and by the initiation of a new, dominant survival programme. Stimulation through TCR antagonized IL-7-/IL-7R-mediated STAT5 activation and Bcl-2 expression, while simultaneously promoting survival through induction of alternative anti-apoptotic Bcl-2 family members, A1 and Bcl-xL. The successful induction of the new survival promoting proteins counteracting the loss of the homeostatic IL-7-/IL-7R-driven survival signals determines whether a cell survives to the first division, allowing it to participate in the immune response. The efficiency of the induction of this new survival programme is dependent on the strength of the antigen/TCR signal. Furthermore, for the transition between the two survival programmes, multiple inputs to the regulation of pro- and anti-apoptotic Bcl-2 family members are integrated to determine the fate of the cell. The strong induction of pro-apoptotic Bim and Bax at the same time as downregulation of Bcl-2 suggests an active inhibition of cell survival after TCR stimulation that needs to be counteracted by the upregulation of the anti-apoptotic Bcl-2 family members A1 and Bcl-xL. However, a substantial number of T cells fail to induce these pro-survival molecules sufficiently after TCR stimulation, and they therefore die before entering the first division. The induction of A1 and Bcl-xL after TCR stimulation requires calcineurin as well as MEK1/2 signalling, whereas the downregulation of STAT5 activation and Bcl-2 expression are independent of these pathways. Consequently, inhibitors of calcineurin (for example, CsA and FK506) or the MEK1/2 pathway block the induction of the new survival programme, but do not affect the inhibition of the TCR stimulation-induced Bcl-2 downregulation. T cells stimulated in the presence of these drugs lose their pre-existing (IL-7-/IL-7R-driven) protection from apoptosis and fail to initiate the alternate (TCR-driven) survival programme, therefore, favouring death over division. Inhibition of either the calcineurin or the MEK pathways leads to inhibition of both proliferation and

survival of T cells after TCR ligation^{31,43–45}. Our findings provide a mechanism of diminishing the proliferative pool of T cells through reduction of survival before cells enter the first division.

The differential expression and interplay of members of the Bcl-2 family of survival-regulating proteins have long been known to be involved in the control of survival in developing naive and mature T cell populations^{46–50}. In this context, the early induction of A1 following TCR ligation has been proposed to release cells from the delay in cell cycle entry that has been observed to result from Bcl-2 overexpression^{37,51}. However, this effect has so far only been observed in T cells from Bcl-2 tg mice, and it is not clear whether physiological levels of Bcl-2 can achieve the same effect. In light of the inhibition of IL-7R signalling that we observed, our results suggest an alternative interpretation, whereby T cells make a commitment to one regulatory survival network or the other: IL-7-/IL-7R-driven or TCR-driven. Commitment to the latter survival programme is reinforced by the inhibitory actions of TCR stimulation on Bcl-2 expression and the induction of pro-apoptotic Bcl-2 family members (for example, Bim and Bax). This results in a ‘clean slate’ for the control of cell survival, creating a T cell that would die rapidly without the introduction of an alternative survival programme. Our data also suggest that the transition is not simply ‘all or none’, but quantitative, as T cell survival varied under different TCR activation conditions and TCR stimulation strengths. The varying ability of high- and low-affinity peptides (strong and weak TCR activators, respectively) and of CD3 antibodies (weak), to promote T cell survival, is consistent with a quantitative mode-of-survival regulation. This would explain why different proportions of T cells die under different conditions (high- versus low-affinity TCR stimulation) before reaching their first division. Consistent with this quantitative view of the transition between the two cell survival programmes, we found that costimulatory signals, such as IL-2, further enhance survival and increase the number of T cells entering the first division, even in the presence of CsA. This model also explains why TCR stimulation can paradoxically lead to a mixture of cell death and cell proliferation at the population level, as was seen here both *in vitro* and *in vivo*.

T cell homeostasis and activation both require the regulation of cell survival and proliferation. Intermittent signalling of IL-7 and TCR were shown to be required for long-term survival to maintain homeostasis of naive T cells⁵². For full activation by

antigen it appears evident that these two regulatory mechanisms should not act in competition, as they could potentially offer conflicting signals to T cells. Here we show that once activated, the T cell must integrate the signals from the TCR and costimulatory receptors that effectively put it into a competition with other T cells, to select the fittest cells, and those best suited for the fight against a particular pathogen. Many weakly stimulated T cells may not survive long enough to enter the first division. This may also be part of a failsafe mechanism to reduce the chances of autoreactive T cells (bearing TCRs that can also bind with low affinity to foreign antigens presented by self-MHC proteins) from being adventitiously expanded and functionally activated into an effector state, which could cause autoimmune tissue destruction. Identifying this fork in T cell fate determination is not only useful in understanding T cell behaviour but may have practical implications. The ability of calcineurin inhibitors (for example, CsA and FK506) to block induction of the new survival programme may explain their ability to promote early deletion of antigen-stimulated T cells³⁰. Treatment with these drugs alone does not appear to be sufficient to delete all antigen-reactive cells, as other receptors, such as the IL-2R, can promote cell survival. Thus, collectively, these studies suggest that blocking several (or maybe even all) sources of survival signals following antigen stimulation in the presence of calcineurin inhibitors may be needed to eliminate antigen-reactive cells from the repertoire, while sparing the unstimulated T cells. Such a form of immunotherapy would be useful to prevent tissue graft rejection or restore self-tolerance during early stages of autoimmune disease.

Methods

Mice. C57BL/6, C57BL/6-Ly5.1, BALB/c, C57BL/6-SOCS-1/IFN- γ , -2, -3, -Bim, -Puma, -Noxa, -Bad, -Bid and -Bim/Puma knock-out and TCR tg mice expressing on their T cells TCRs specific for chicken ovalbumin—OT-I, OT-II and DO11.10—were obtained from the Walter and Eliza Hall Institute (WEHI, Parkville, VIC, Australia) animal facility (Kew, VIC, Australia). Mice were maintained under specific pathogen-free conditions and used between 5 and 12 weeks of age. All animal experiments were performed under the approval from the WEHI Animal Ethics Committee.

Cell preparation and *in vitro* cell culture. CD4⁺ or CD8⁺ T cells were isolated from lymph nodes or spleens using the CD4 + CD62L + T Cell Isolation Kit II or the CD8a + T Cell Isolation Kit II, respectively, from Miltenyi Biotech. For cell survival and proliferation assays, purified CD4⁺ or CD8⁺ T cells were labelled with 2.5 μ M CFSE (Invitrogen) as previously described⁵³. In some experiments, CFSE was replaced with 5 μ M Cell Trace Violet (CTV) (Invitrogen) and cells labelled according to the manufacturer's instructions. Cells were cultured in RPMI 1640 medium supplemented with non-essential amino acids, 1 mM N-pyruvate, 10 mM HEPES, 100 U ml⁻¹ Penicillin, 100 μ g ml⁻¹ Streptomycin (all Invitrogen), 50 μ M 2-mercaptoethanol, 2 mM L-glutamine (both Sigma) and 10% FCS (JRH Biosciences).

T cells were plated in flat-bottom (CD3 antibody stimulation) or round-bottom (peptide and peptide-coated APC stimulation) 96-well plates. T cell culture for western blot or mRNA analysis was done in 24-well plates or T75 tissue-culture flasks. rIL-2 (100 U ml⁻¹; a gift from G. Zurawski, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) and 1 ng ml⁻¹ (unless otherwise indicated) rIL-7 (Peprotech) were added to the culture where indicated. ABT-737 (Abbott Laboratories), CsA (Cell Signalling Technologies), U0126 (Selleck Chemicals), Ly294002 (Calbiochem) and Bisindolylmaleimide (Sigma) were added at the concentrations indicated. For CD3 antibody stimulation, tissue-culture wells were coated either at 37 °C for 2 h or at 4 °C overnight with 10 μ g ml⁻¹ anti-CD3 antibody (clone 145-2C11) before culture. For peptide stimulation of OT-I cells, the MHC class I presentable OVA-derived peptide SIINFEKL (N4) (OVA₂₅₇₋₂₆₄) or variants with amino acid exchanges in position 4 or 7 (V4, G4, S4, D7; Auspep) were added at the concentrations indicated, directly to the culture medium for stimulation of OT-I cells through self-presentation²⁴. CD4⁺ T cells from OT-II and DO11.10 TCR tg mice were stimulated by autologous splenocytes (used as APC with T cell to splenocyte ratio 1:40) and 1 μ g ml⁻¹ of the MHC class II presentable OVA-derived peptide ISQAVHAHAHAEINEAGR (OVA323-339) (JPT Peptide Technologies). Cell cultures were incubated at 37 °C with 5% CO₂ in a humidified atmosphere.

***In vivo* experiments.** Purified and CTV-labelled CD8⁺ T cells, 8×10^5 (Exp 1, Fig. 7a) or 5.6×10^5 (Exp 2, Fig. 7b, c), from OT-1-Ly5.2 mice were injected into the tail vein of C57BL/6-Ly5.1 mice 22 h (Exp 1) or 27 h (Exp 2) before commencement of treatment. FK506 (2.5 mg kg⁻¹; Prograf; Janssen-Cilag) diluted in saline or only saline (used as a negative control) were injected intraperitoneally every 6 h throughout the duration of the experiment. Ten microgram SIINFEKL peptide diluted in saline or only saline (used as a negative control) were injected intravenously 1 h (Exp 1) or 2 h (Exp 2) after the first FK506 injection. At the time points indicated, spleen and lymph node (axillary, brachial, inguinal and mesenteric) were collected, gently meshed up, passed through 70- μ m cell strainers to produce a single-cell suspension and stained with surface marker-specific antibodies for flow cytometric analysis. CD8⁺ OT-I cells were identified as CD8⁺, LY5.2⁺, TCR α 2⁺ and CTV⁺.

Flow cytometry and cell counting. Flow cytometric analysis was performed on a FACSCanto II flow cytometer (BD Biosciences) and data were analysed using FlowJo (Tree Star). For cell counting, 10⁴ cells per well were placed in 96-well plates at the beginning of the culture. A known number of bead particles (Flow Check Rhodamine Low-Brite Beads (Polysciences)) and 0.2 μ M propidium iodide (Sigma) for dead cell exclusion were added to each sample immediately before analysis. The live cell number per sample was then determined by the ratio of live cells to beads.

Monoclonal antibodies for detection of the following cell surface markers were used: IL7-R α chain-R-PE, CD4-PerCP, CD8-APC-Cy7, CD8-FITC, TCR α 2-R-PE, TCR α 2-biotin (revealed by FITC-coupled streptavidin (Caltag)) and CD45.2 (Ly5.2)-APC (all from BD Biosciences).

For intracellular phospho-STAT-5 staining, cells were collected, fixed and permeabilized using Phosphoflow buffers following the manufacturer's protocols, followed by staining with Stat5 (pY694) antibodies coupled to Alexa488 (all BD Biosciences).

Western blot analysis. At indicated time points, cells were collected, washed 1 \times with PBS and the frozen at -80 °C. Cell pellets were lysed in lysis buffer (KALB (1% (vol/vol) Triton X-100 in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM EDTA supplemented with 2 mM sodium vanadate, 10 mM NaF, complete protease inhibitor mixture (Roche Applied Bioscience) and 1 mM PMSF) and equal amounts of lysate were separated on 4–12% Bis-Tri gels (Bio-Rad) and reducing conditions and blotted onto PVDF membranes (Osmonics). Primary antibodies for detection of the following proteins used were hamster anti-Bcl-2, rat-anti-Bim, mouse-anti-Bax, (all WEHI antibody facility), rat-anti-A1 (clone 6D6-1-1, MJ Herold, manuscript in preparation), rabbit-polyclonal-anti-Bcl-xL (BD Transduction Laboratories), goat-anti-Actin-HRP (Santa Cruz), mouse-anti-pSTAT5 (Tyr694/699) (Upstate, Millipore), rabbit-anti-pJAK1 (Tyr1022/1023) (Invitrogen) and mouse-anti-STAT5a (Invitrogen), followed by incubation with secondary antibodies goat-anti-rat-Ig-HRP (SouthernBiotech), goat-anti-hamster-Ig-HRP (SouthernBiotech), donkey-anti-rabbit-Ig-HRP or sheep-anti-mouse-Ig-HRP (both GE Healthcare). Proteins were revealed using ECL Luminata Forte reagent (Millipore).

RNA isolation and real-time PCR analysis. Total RNA was isolated by using the RNeasy Kit including DNase digestion step (Qiagen) and transcribed to cDNA using High Capacity RNA to cDNA Mastermix (Applied Biosystems), all following the manufacturer's instructions. Reverse transcription-PCR was performed using Power SYBR green Mastermix on an ABI-PRISM 7900 thermal cycler (all Applied Biosystems). Relative mRNA level of samples relative to freshly isolated cells was quantified using the $\Delta\Delta$ Ct-method. The primers used were as follows:

a1 (5'-GTCATACTTGGATGACTTTCACGTG, ATTCTCCTGTGTTATTCAT TATGAATTCTG-3');
bcl-2 (5'-TTATAAGCTGTACAGAGGGGCTAC, GAACTCAAAGAAGGCC ACAA TCCTC-3');
bcl-xL (5'-TGGAGTCAGTTTAGTGATGTGCAAG, AGTTTACTCCATCCCC AAAGAGTTC-3');
mcl-1 (5'-GAGGAGGAAGAGGACGACCTATACC, AGTTTCTGCTAATGGT TCGATGAAG-3'); and
 β -actin (5'-TATTGGCAACGAGCGGTTTC, CCATACCCAAGAAGGAAGGCT-3')

Statistical analysis. One-way analysis of variance was applied to test the effect of different treatments. Log-transformed numbers were used with Tukey's post-tests for comparing all groups.

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Author contributions

P.K., S.H. and P.D.H. designed the study; P.K., S.H., E.M.C. and J.G.Z. performed experiments and analysed data; L.H., W.S.A., C.L.S., A.M.L., M.J.H. and A.S. provided mice as well as reagents, and gave conceptual advice; S.H., P.K. and P.D.H. wrote the paper. P.D.H. led the research programme. S.H. and P.K. contributed equally to this work and are joint first authors.

Additional information

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