Loss of c-Cbl RING finger function results in high-intensity TCR signaling and thymic deletion

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Signaling from the T-cell receptor (TCR) in thymocytes is negatively regulated by the RING finger-type ubiquitin ligase c-Cbl. To further investigate this regulation, we generated mice with a loss-of-function mutation in the c-Cbl RING finger domain. These mice exhibit complete thymic deletion by young adulthood, which is not caused by a developmental block, lack of progenitors or peripheral T-cell activation. Rather, this phenotype correlates with greatly increased expression of the CD5 and CD69 activation markers and increased sensitivity to anti-CD3-induced cell death. Thymic loss contrasts the normal fate of the c-Cbl^{-/-} thymus, even though thymocytes from both mutant mice show equivalent enhancement in proximal TCR signaling, Erk activation and calcium mobilization. Remarkably, only the RING finger mutant thymocytes show prominent TCR-directed activation of Akt. We show that the mutant c-Cbl protein itself is essential for activating this pathway by recruiting the p85 regulatory subunit of PI 3-kinase. This study provides a unique model for analyzing high-intensity TCR signals that cause thymocyte deletion and highlights multiple roles of c-Cbl in regulating this process.

The EMBO Journal (2005) 24, 3807–3819. doi:10.1038/sj.emboj.7600841; Published online 6 October 2005

Subject Categories: immunology

Keywords: Akt; apoptosis; Cbl; CD3; thymus

Introduction

The generation of T cells that respond to foreign antigens, but not self-antigens, is carried out in the thymus by a process initiated by T-cell receptor (TCR) engagement and the activation of intracellular signaling cascades. The amplitude and duration of these signaling responses are initially determined by the affinity of the TCR for antigen/MHC complexes and the total number of receptor interactions. After antigen engagement, signaling pathways are controlled by an array of

intracellular enzymes, regulatory proteins, adaptors and transcription factors. The strength and kinetics of these signaling responses are key factors determining whether thymocytes survive or are actively deleted, by positive and negative selection respectively (Ohashi, 2003; Palmer, 2003; Starr *et al*, 2003). Perturbations to signaling molecules functionally involved in these outcomes can alter the fate of thymocytes and result in the development of anergy or autoimmunity. Thus, the ability to generate a functional T-cell repertoire is of great importance and requires precise regulation of ligand engagement and signal transduction.

A key regulator of TCR and CD3 levels, and the activity of signaling proteins downstream, is c-Cbl (Thien and Langdon, 2001; Liu and Gu, 2002; Dikic *et al*, 2003; Liu, 2004). c-Cbl, and its close homologue Cbl-b, principally functions as an E3 ubiquitin ligase by virtue of a RING finger domain, which recruits ubiquitin conjugating enzymes (E2s), and a tyrosine kinase binding (TKB) domain involved in substrate targeting. The best-characterized substrates for c-Cbl- and Cbl-b-directed ubiquitylation are receptor tyrosine kinases; however, other classes of receptors, cytoplasmic protein tyrosine kinases (PTKs), adaptor proteins and regulatory proteins have also been identified as targets. Studies of c-Cbl knockout (KO) mice found elevated TCR and CD3 levels on the surface of CD4⁺CD8⁺ double positive (DP) thymocytes, increased levels of the Src family kinases Lck and Fyn and increased activity of the ZAP-70 tyrosine kinase (Murphy *et al*, 1998; Naramura *et al*, 1998; Thien *et al*, 1999). Indeed, CD3 signaling in c-Cbl KO thymocytes is enhanced to an extent where ZAP-70 activation is uncoupled from a requirement for CD4 coreceptor ligation, although this effect is independent of TKB domain function (Thien *et al*, 1999, 2003). Despite these perturbations that increase the intensity and duration of TCR signals, c-Cbl KO thymi develop with apparent normality. However, the absence of c-Cbl enhanced positive selection of CD4⁺ thymocytes in MHC class II-restricted TCR transgenic mice (Naramura *et al*, 1998). These findings are consistent with roles for c-Cbl in negatively regulating TCR signals involved in determining the fate of thymocytes.

To better understand the mechanisms involved in this regulation, we generated mice with a loss-of-function mutation in the c-Cbl RING finger domain. This substitution of an alanine for the amino-terminal cysteine in the C₃HC₄ RING domain at position 379 (C381 in human c-Cbl) has been well characterized and abolishes c-Cbl's interaction with E2s and its function as an E3 ubiquitin ligase (Joazeiro *et al*, 1999; Levkowitz *et al*, 1999; Ota *et al*, 2000; Thien *et al*, 2001). Unlike the mouse with a loss-of-function mutation in the c-Cbl TKB domain (Thien *et al*, 2003), we find that the RING finger mutant mouse resembles the c-Cbl KO in many respects, such as equivalently enhanced levels of CD3, TCR and Lck in DP thymocytes. However, the RING finger mutation induces additional phenotypic changes that are more severe than those observed in c-Cbl KO mice, notable among these being a progressive loss of the thymus.

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Results

Generation of mice with a loss-of-function mutation in the c-Cbl RING finger

Mice with a Cys to Ala substitution at position 379 (C379A) were generated and genotyped as outlined in Figure 1A and B. Matings of heterozygous C379A c-Cbl mice (termed +/A) produced significantly less than expected homozygous mutant (A/A) offspring (5% compared to expected 25%; Figure 1C) with the majority of these dying *in utero* after E14 (22% A/A detected at E14, 7% at E16 and 4% at E19). In addition, ~25% of A/A mice born did not survive the first

24 h (Figure 1C). Interestingly, this severe developmental effect does not occur in either the c-Cbl or Cbl-b KO mice. To overcome the scarcity of A/A mice, we generated c-Cbl A/– mice. These had improved, although still reduced, viability (Figure 1C), indicating that a single copy of the mutated allele has a less severe effect on survival. Importantly, c-Cbl A/– mice appear to be indistinguishable from homozygous c-Cbl A/A mice for all other phenotypic perturbations identified to date.

Western blotting showed that c-Cbl protein levels in thymocytes are not affected by the C379A mutation (Figure 1D). Interestingly, A/A and A/– mice had darker coats, feet and

Figure 1 Generation and identification of c-Cbl(C379A) mice. **(A)** Genomic organization of the mouse c-Cbl gene showing the region targeted for homologous recombination to introduce the C379A mutation (indicated by a large asterisk). Targeted ES cell clones were identified by Southern blotting using 5' and 3' probes as indicated. B, BamHI; H, HindIII; X, XbaI; X*, XbaI sites present in the 129Sv/J but not C57BL/6 strain. The loxP-flanked pGKNeo cassette was removed by Cre-mediated excision *in vivo*, leaving a single loxP site (solid triangle). **(B)** PCR genotyping of c-Cbl(C379A) mice prior to Cre-mediated deletion using primers p1 and p2 to detect the wt allele (~600 bp product), and p1 and p3 to detect the C379A targeted allele (~450 bp product). **(C)** Genotype frequencies and survival statistics. The percentage of pups of each genotype that die within the first 24 h of birth is also shown. **(D)** Expression of the C379A allele does not alter c-Cbl protein levels. Thymocyte lysates from c-Cbl +/A, A/A, A/– and +/– mice were immunoblotted with antibodies to c-Cbl, ZAP-70 or Erk1/2. **(E)** Expression of the C379A allele enhances coat color in black and agouti mice. Comparisons of a black c-Cbl(C379A) homozygous mutant mouse (A/A) with a wt C57BL/6 mouse (left panel), c-Cbl +/+ and A/A agouti littermates (middle), and agouti c-Cbl –/–, A/A and A/– mice (right) show that c-Cbl(C379A) knockin mice have darker coats, paws, ears and tails than wt or c-Cbl KO mice.

tails than wild-type (wt) or KO mice (Figure 1E). This phenotype occurred in both black and agouti mice and is not evident in other Cbl mutant mice generated to date. The mechanism for the dark coloration was not examined but may be linked to enhanced activity of c-Kit, which is negatively regulated by Cbl proteins (Zeng *et al*, 2005) and is required for melanocyte development.

Progressive thymic loss in the c-Cbl RING finger knockin mouse

Examination of organs revealed a striking phenotype of c-Cbl C379A mice, namely the progressive loss of thymi as they approach adulthood. In the few A/A mice analyzed, a decrease in thymus size was evident by 2 weeks while a 40-day-old A/A mouse contained fewer than 1% of total thymocytes compared to its +/A littermate (Figure 2A). Comparison of over 100 A/- and +/- littermates shows that this progressive loss of the thymus was similarly observed in A/- mice (Figure 2B and C). This phenotype was unexpected since c-Cbl KO mice do not show this thymic loss and indeed exhibit a slight increase in thymic cellularity as young adults (Murphy *et al*, 1998).

A consequence of thymocyte loss in RING finger mutant mice was that lymph nodes contained 50% fewer CD4⁺ and CD8⁺ T cells and a higher proportion of B cells compared to normal littermates and c-Cbl-/- mice (Supplementary Figure 1A and B). A greater proportion of CD4⁺ T cells expressing higher levels of the activation markers CD44 and CD25 was evident in A/- mice; however, the absolute number of cells with this phenotype was decreased and CD62L levels, a marker for memory T-cell populations, were equivalent between CD4⁺ T cells from c-Cbl-/- and A/- mice (Supplementary Figure 1C). Spleens from A/A and A/- mice were increased 2.5- to 3.5-fold in size compared to a 1.2- to 2-fold increase in c-Cbl-/- mice and is caused by a greater expansion of the red pulp with increased numbers of red blood cells, megakaryocytes, megakaryoblasts and myelocytes (Figure 2B and C and data not shown). However, there is no evidence of lymphocyte hypertrophy in A/- spleens and indeed the proportion of splenic T cells is generally reduced by 80% compared to normal littermates analyzed between 4 and 7 weeks of age.

Thymic loss is not due to a developmental block

The effect of the C379A mutation in inducing thymic loss prompted us to investigate whether this was due to a block in thymocyte development. However, representative analyses of CD4 and CD8 expression on C379A knockin thymocytes detected double negative (DN), DP and single positive (SP) populations in near-normal proportions (Figure 2D). Thus, there is no major block in the DN to DP transition, or in the selection of DP to SP thymocytes. We observed slight increases in the proportion of DN thymocytes from RING finger mutant mice (Figure 2E), which became pronounced when thymic loss was greatest and was accompanied by a corresponding decrease in the proportion of DP thymocytes (Figure 2F). However, analysis of A/- thymi using CD44 and CD25 antibodies revealed no marked effects on the four major developmental stages of the DN population, aside from a tendency toward an increased proportion of DN2/3 cells in older mice (Figure 2G).

The proportion of mature CD4 and CD8 SP thymocytes was also reduced, by approximately 40% (Figure 2D and E). This suggests that the C379A mutation also perturbs signaling events that determine SP selection, presumably because of changes to TCR and coreceptor signal strength.

Thymic loss is not due to limiting numbers of progenitors

We also investigated whether thymic loss was due to limiting numbers of progenitors reseeding the thymus. This was tested by repopulating lethally irradiated B6 CD45.1 congenic mice with bone marrow from wt (CD45.1) and c-Cbl +/-, A/- or -/- (CD45.2) donors mixed in 1:1 or 4:1 ratios. Mice reconstituted with marrow from single donors confirmed that >98% repopulation of the thymus with donor progenitors occurs by 3 weeks post-transfer. At this time, thymi of all reconstituted mice were of a similar size (Figure 3A); however, by 4 weeks, the mouse receiving A/- marrow alone had 59 and 80% fewer thymocytes than recipients of wt or c-Cbl-/- marrow, respectively (data not shown). By 5 weeks, thymic deletion was nearly complete, with the A/- recipient having only 4×10^6 thymocytes in contrast to 268×10^6 and 334×10^6 thymocytes in recipients of wt and c-Cbl-/- marrow, respectively (Figure 3A).

In mixed bone marrow experiments, the relative contribution of each donor reconstituting the DP thymocyte population was determined by anti-CD45.1 (+/+) and anti-CD45.2 (A/- or +/-) staining. From such experiments, it was clear that A/- thymic progenitors were not limiting and indeed could repopulate the thymus with greater efficiency than either c-Cbl +/+, +/- or -/- marrow (Figure 3B and data not shown). A time-course analysis showed that at 3 weeks after transfer, equivalent contributions were evident among all groups receiving the 1:1 mixes (Figure 3B, first three panels, top row). Remarkably, by 4 weeks, 99% of thymocytes in the 1:1 mix of +/+ and A/- marrow originated from the A/- donor (Figure 3B), although the thymus had not yet diminished in size compared to the recipient of the +/+: +/- mix (188 and 194×10^6 thymocytes, respectively). Thymic depletion became apparent at 5 weeks, indicating that the A/- contribution had completely overwhelmed the +/+ contribution to the extent that the +/+ thymocytes did not have an opportunity to rescue the thymus (Figure 3A, lower middle panel). The competitive advantage of A/- marrow is shown even more clearly when the repopulating mix was biased 4:1 in favor of +/+ (CD45.1) marrow (last two columns of Figure 3B). This slowed but did not prevent the dominance of A/- thymocytes, with a 95% contribution from the c-Cbl A/- donor detected after 5 weeks (lower right panels of Figure 3B) at which time the thymus was reduced 50% in size compared to the 4:1 +/+: +/- control. Thus, even when diluted four-fold, the A/- thymocytes were able to outcompete +/+ thymocytes and prevent thymic rescue.

These results demonstrate that thymic progenitors are not limiting in the c-Cbl A/- mouse and that thymic deletion is due to an inherent perturbation of c-Cbl A/- thymocytes, and not from an altered stroma. The marked repopulating bias by A/- marrow may be a property of multipotential progenitors since similar increases in A/- derived cells were seen in the B lymphoid and myeloid lineages (Supplementary Figure 2 and data not shown). The prominence of A/- thymocytes

does not appear to be due to a growth advantage, as all three genotypes showed equivalent numbers of BrdU-positive thymocytes after injection with APC-labeled BrdU (data not shown). Similarly, cell cycle analysis of wt, c-Cbl A/- and -/- thymocytes revealed similar proportions in S and G2/M phases (data not shown).

Thymic loss is not due to peripheral T-cell activation

Peripheral T-cell activation can cause nonspecific thymocyte death by eliciting a 'cytokine storm' (Martin and Bevan, 1997; Brewer *et al*, 2002; Zhan *et al*, 2003). To determine if this is the cause of thymic loss in the c-Cbl A/- mouse, we transferred c-Cbl A/- bone marrow into lethally irradiated

GK/2.43 mice, which lack peripheral T cells. GK/2.43 mice are doubly transgenic for anti-CD4 (GK1.5) and anti-CD8 (2.43) antibodies that deplete CD4⁺ and CD8⁺ T cells in the periphery yet do not affect thymocyte development (Zhan *et al*, 2000b, 2003; Y Zhan and AM Lew, unpublished). Analysis of mice 3, 4 and 5 weeks after transfer showed that thymic deletion progressed with equivalent kinetics and severity in recipient GK/2.43 mice as that of control B6.CD45.1 mice that received A/– marrow (Figure 3C).

Importantly, the GK/2.43 recipient mice lacked splenic or lymph node T cells, whereas T cells were evident in the B6.CD45.1 recipients (Figure 3D).

Thymocytes from the C379A mouse are susceptible to anti-CD3-induced death

Since thymic deletion cannot be explained by a developmental block, a lack of progenitors or peripheral T-cell activation,

Figure 3 Thymocyte loss is not due to limiting numbers of progenitors. **(A)** Thymi from irradiated mice 3 and 5 weeks after bone marrow transfer showing thymic loss in irradiated mice receiving c-Cbl A/– bone marrow, either alone or as a mix with wt marrow. **(B)** Flow cytometric profiles of CD4⁺ CD8⁺ DP thymocytes from lethally irradiated mice 3, 4 and 5 weeks after mixed bone marrow reconstitution. The contribution to DP thymocytes from wt donor marrow was determined by CD45.1 staining and the contribution from c-Cbl +/–, A/– or –/– marrow by CD45.2 staining. Similar proportions of donor contribution were observed in DN and SP populations. **(C)** Thymi from irradiated B6.CD45.1 or GK/2.43 transgenic mice 3 and 5 weeks following bone marrow reconstitution show that equivalent thymic loss in A/– bone marrow recipients occurs in the presence (B6.CD45.1) or absence (GK/2.43) of peripheral T cells. The number of thymocytes ($\times 10^6$) isolated from each thymus are shown. **(D)** Flow cytometric profiles of cell surface TCR on spleen and mesenteric lymph node cells showing the absence of peripheral T cells in GK/2.43 reconstituted mice 5 weeks after bone marrow transfer.

Figure 2 Thymocyte loss in the c-Cbl(C379A) knockin mouse is not caused by a developmental block. **(A)** Total thymocyte numbers from five pairs of c-Cbl A/A and +/A littermates at various ages. The difference is tabulated as the percentage decrease in thymocyte numbers from the c-Cbl A/A mouse compared to its +/A littermate. **(B)** Photograph and weights of spleens and thymi showing the greatly reduced thymi but enlarged spleens in c-Cbl A/– mice compared to their +/– littermates. **(C)** Total thymocyte numbers and spleen weights of c-Cbl A/– (red circles) and +/– littermates (black triangles) killed between 1 and 10 weeks of age. **(D)** Flow cytometric analysis of CD4 and CD8 on thymocytes from 19- and 36-day-old c-Cbl +/–, A/– or –/– mice. Percentages of DN, DP, and CD4 or CD8 SP populations are indicated in the respective quadrants. **(E)** Bar graph representing the mean percentages ($\pm$ SEM) of CD4 and CD8 DN, DP and CD4 or CD8 SP thymocytes from 23 c-Cbl +/– and 28 A/– littermates. Statistically significant differences between A/– and +/– DN, CD4 and CD8 SP populations were detected using unpaired *t*-test (***P* < 0.05; ****P* < 0.001). **(F)** Percentages of DN and DP thymocytes from +/– and A/– littermates of varying thymus size. The data reveal that the smallest A/– thymi show reduced proportions of DP thymocytes and a corresponding increase in the DN population. **(G)** Flow cytometric analysis of DN subpopulations. Thymocytes from 19-, 28- and 36-day-old c-Cbl A/– and +/– littermates were stained with FITC-conjugated antibodies to CD4, CD8, CD3, TER119, B220 and Gr1, PE-conjugated anti-CD25 and APC-conjugated anti-CD44. Analysis of CD44 and CD25 expression was performed on gated FITC-negative cells. The percentage of cells found in each quadrant is indicated.

we investigated the possibility that RING finger mutant thymocytes are more susceptible to CD3-directed death signals. Induction of thymocyte apoptosis *in vitro* requires triggering of both CD3 and CD28 receptors, and this response is confined to the DP population and is not dependent on Fas or TNF receptor interactions (Punt *et al*, 1997). Consistent with this, thymocytes from all three genotypes exhibited cell death when exposed to anti-CD3 + CD28 antibodies (Figure 4A). However, c-Cbl A^{-/-} thymocytes cultured with anti-CD3 also invariably showed marked induction of cell death compared to those from c-Cbl +/− littermates, which did not die in response to this level of *in vitro* stimulation (Figure 4A, data from five experiments). The c-Cbl KO thymocytes were also susceptible to anti-CD3-induced cell death but at approximately half the level of that observed for c-Cbl A^{-/-} thymocytes. Thus, a signaling response through CD3 alone in c-Cbl RING finger mutant thymocytes is of sufficient intensity to induce cell death. The uncoupled requirement for coreceptor signals in thymocytes and T cells is a common theme in Cbl mutant mice and these findings provide another example where a Cbl mutation promotes greater responsiveness to a suboptimal signal.

Thymocyte loss in the C379A mouse is rescued by a Bcl-2 transgene

We reasoned that if the thymic loss in C379A mice involved a cell death mechanism, then this phenotype would be abrogated by expression of a prosurvival molecule. Indeed, using Vav promoter-driven Bcl-2 transgenic mice (Ogilvy *et al*, 1999), we found that Bcl-2 expression in C379A mice can rescue *in vivo* thymic loss (Figure 4B, upper table) and block anti-CD3 mediated thymocyte death *in vitro* (Figure 4B, lower panels). In the light of these findings, we examined levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L, as well as two proapoptotic proteins of the Bcl-2 family, Bim and Bax (Strasser, 2005). However, analysis of these four family members in c-Cbl +/−, A^{-/-} and −/− thymocytes revealed no gross changes in their levels (Figure 4C) although a compromise in their function cannot be ruled out.

Equivalent CD3 and TCR levels on c-Cbl C379A and c-Cbl KO DP thymocytes

Increased levels of CD3 and TCR on DP thymocytes from c-Cbl−/− mice contribute to enhanced signal strength in these cells (Murphy *et al*, 1998; Naramura *et al*, 1998; Thien *et al*, 1999). Higher levels of these receptors in the C379A mouse could expose cells to stronger signals and possibly lead to a proapoptotic response. However, remarkably equivalent increases were seen in CD3 and TCR levels on c-Cbl−/− and A^{-/-} DP thymocytes compared to wt controls (Figure 5A). This clearly demonstrates that the increased levels of these receptors on c-Cbl−/− DP thymocytes are specifically caused by a loss of RING finger function. This finding also indicates that thymic deletion in the RING finger mutant mouse cannot be explained by antigen receptor levels on DP thymocytes exceeding those of the c-Cbl−/− mouse. Furthermore, repeated analyses showed no difference between both mutant thymocytes in the kinetics of CD3 or TCR internalization, the loss of these receptors following internalization, or TCR recycling (Supplementary Figure 3

Figure 4 A Bcl-2 transgene rescues the c-Cbl(C379A) thymus. (A) c-Cbl(C379A) thymocytes show enhanced susceptibility to *in vitro* anti-CD3-mediated cell death. Bar graphs represents the mean percentages ($\pm$ SEM) from five experiments of PI-positive thymocytes above that of unstimulated controls following 24 h culture with plate-bound anti-CD3 or anti-CD3 + CD28. c-Cbl +/− (white bars), A/− (black) and −/− (hatched). Statistically significant differences as measured by unpaired *t*-test are indicated: ***P* < 0.001 and ****P* < 0.0001. The extent of cell death in unstimulated thymocyte cultures from wt, c-Cbl A/− and c-Cbl −/− mice was not significantly different. (B) The Bcl-2 transgene rescues thymic loss and anti-CD3 killing of A/− thymocytes. Four littermates of genotypes c-Cbl +/−, c-Cbl A/−, c-Cbl +/−;Vav-Bcl-2 and c-Cbl A/−;Vav Bcl-2 were examined for thymocyte numbers and *in vitro* killing by plate-bound anti-CD3. Dead cells were detected by flow cytometry for uptake of PI. (C) Levels of pro- and antiapoptotic proteins are not altered in c-Cbl mutant thymocytes. Thymocyte lysates from 3- and 4-week-old mice were immunoblotted with anti-Bim, Bax, Bcl-2 and Bcl-x_L antibodies. The greater intensity in 4 weeks lysates is due to loading differences and not an age-related effect.

and data not shown). Therefore, the opposing thymic outcomes in these two mutant mice cannot be attributed to differences in ligand-induced internalization or processing of the receptor that might affect the duration of signaling.

Figure 5 Increased expression of surface TCR, CD3, CD5 and CD69 on DP thymocytes from c-Cbl mutant mice. DP thymocytes from age-matched sets of mice at 10 days, 3 weeks or 5 weeks were analyzed by flow cytometry for cell surface expression of TCR, CD3, CD5 and CD69. c-Cbl $+/+$ (shaded histogram), A/ $+$ (bold) and $-/-$ (dashed).

CD5 and CD69 expression is greatly increased in RING finger mutant mice

CD5 expression on DP thymocytes parallels the affinity of the positively selecting TCR-MHC-ligand interaction, suggesting that its expression fine-tunes the strength of the TCR signaling response (Tarakhovsky *et al*, 1995; Azzam *et al*, 1998). CD69 is another marker of thymocyte activation and its upregulation occurs during both positive and negative selection (Bendelac *et al*, 1992; Kishimoto and Sprent, 1997). Consistent with this, enhanced signaling from the TCR is accompanied by increased surface expression of CD5 and CD69 on DP thymocytes from the c-Cbl KO mouse (Naramura *et al*, 1998; Thien *et al*, 2003). Remarkably, levels of CD5 and CD69 are even more elevated in the RING finger mutant mouse (Figure 5). This increase becomes more marked with age (compare 5 weeks with 10 days), suggesting that the majority of the DP cells remaining in the diminishing thymus have encountered very strong TCR-directed signals. Indeed, the enhanced upregulation of CD5 and CD69, despite similar levels of TCR and CD3 on c-Cbl A/ $+$ and c-Cbl $-/-$ DP thymocytes, indicates that a more potent signal is being transmitted downstream of the TCR/CD3 complex in the A/ $+$ thymus.

Thymic loss is not linked to enhanced ZAP-70 or ERK activation

Consequently, we sought to determine whether there is evidence of differentially enhanced signaling downstream of the antigen receptor that could account for thymic loss in the C379A knockin, but not the c-Cbl KO. Crosslinking of CD3

and CD4 rapidly induces tyrosine phosphorylation of Fyn, Lck, ZAP-70, LAT and SLP-76 and this signal is enhanced in c-Cbl $-/-$ thymocytes compared to the wt (Murphy *et al*, 1998; Naramura *et al*, 1998; Thien *et al*, 1999). Consistent with the effects on receptor levels, we observed that c-Cbl A/ $+$ and $-/-$ thymocytes showed equally enhanced protein tyrosine phosphorylation (Figure 6A) and associations between Lck and ZAP-70 (Figure 6B). Lck and Fyn levels were also elevated in thymocytes from both mutant mice (Supplementary Figure 4), providing definitive evidence that these kinases are negatively regulated by the c-Cbl RING finger. Inactivation of the RING finger did not however affect levels of ZAP-70, SLP-76, LAT, Akt, Erk, PLC γ 1, p85 or Cbl-b (Figures 6 and 7 and data not shown).

Although the phosphorylation of proteins in the size range of Src family kinases (50–60 kDa) and LAT appear to be enhanced in c-Cbl A/ $+$ thymocytes in Figure 6A, this was not always observed and in most experiments they were equivalent to the KO. However, c-Cbl A/ $+$ thymocytes invariably showed a transient peak of SLP-76 phosphorylation compared to a more sustained response in c-Cbl KO thymocytes (compare A/ $+$ with $-/-$ after 5 min of stimulation at 37°C in Figure 6A and B).

The JNK and p38 MAP kinase pathways have been implicated in the deletion of DP thymocytes (Rincón *et al*, 1998; Sugawara *et al*, 1998); however, neither of these two proteins showed enhanced activation in thymocytes from either c-Cbl $-/-$ or A/ $+$ mice (data not shown). Furthermore, it is unlikely that ERK signaling is involved in the loss of thymocytes in the RING finger mutant mouse since the amplitude and duration of ERK activation were equally enhanced in both mutants (Figure 6A and C).

RING finger mutant thymocytes show greatly increased levels of phospho-Akt

We did however identify one striking difference in downstream signaling between the c-Cbl mutant mice. Phosphorylation of Akt in A/ $+$ thymocytes was profoundly increased following stimulation and was sustained for at least 30 min (Figure 6A and C and data not shown). This effect was not age dependent (Figure 6C) and therefore not linked to the degree to which the thymus had diminished. Importantly, unlike other signaling molecules examined, this striking enhancement in phospho-Akt was specific to the A/ $+$ mouse, and was not observed in the c-Cbl KO even though Erk activation was similarly enhanced in both.

Enhanced PLC γ 1 activation and calcium mobilization in c-Cbl mutant mice

Akt phosphorylation is a readout of phosphoinositide 3-kinase (PI3K) activation, suggesting that PI3K activity may be elevated in c-Cbl A/ $+$ thymocytes. PI3K can also regulate the phospholipase C (PLC) γ 1/calcium pathway. So we sought to determine if TCR signaling to PLC γ 1 was also differentially affected by measuring the level of phosphorylation on Y83. Stimulation of c-Cbl A/ $+$ and $-/-$ thymocytes triggered an equally enhanced and sustained activation of PLC γ 1, and a correspondingly stronger calcium response, compared to wt thymocytes (Figure 6D and E). Thus in A/ $+$ thymocytes, enhanced signaling appears selective for the PI3K/Akt pathway and is not reflected in the PI3K/PLC γ 1/calcium pathway.

Figure 6 Enhanced signaling in c-Cbl mutant mice. **(A)** Lysates from unstimulated or anti-CD3 + CD4-stimulated thymocytes from c-Cbl +/+ and A/+ littermates, and an age-matched c-Cbl -/- mouse were immunoblotted with antibodies to phosphotyrosine, c-Cbl, ZAP-70, pAkt (pS473), Akt, p-Erk (pT202/pY204) or Erk. A similar enhancement in pAkt from stimulated A/+ thymocytes was also observed using antibodies to pThr308. Positions of c-Cbl, SLP-76, ZAP-70 and LAT are indicated on the anti-phosphotyrosine immunoblot (top panel). **(B)** Unstimulated or anti-CD3 + CD4-stimulated thymocyte lysates from c-Cbl +/+ and A/+ littermates, and an age-matched c-Cbl -/- mouse were immunoblotted with pSLP-76 (pY145) and SLP-76 antibodies (bottom panels) or immunoprecipitated with ZAP-70 antibodies before immunoblotting with anti-phosphotyrosine, anti-ZAP-70 and anti-Lck (upper panels). **(C)** Enhanced phosphorylation of Akt in c-Cbl A/+ thymocytes is independent of age. Thymocytes from 2-, 5- or 6-week-old c-Cbl +/A, +/+, A/+ and -/- mice were left unstimulated or stimulated by anti-CD3 + CD4 crosslinking at 37°C for 5 min. Akt and Erk activation was detected using phospho-specific antibodies and protein levels determined by immunoblotting with Akt and Erk antibodies. **(D)** Enhanced and sustained PLCγ1 activation in c-Cbl mutant mice. Thymocyte lysates from c-Cbl +/+ and A/+ littermates and an age-matched c-Cbl -/- mouse were immunoblotted with pPLCγ1 (pY783) or PLCγ1 antibodies. **(E)** Enhanced and sustained calcium mobilization in c-Cbl mutant mice. Fluo-4-loaded thymocytes from c-Cbl +/+ (dashed), A/+ (black bold) and -/- (gray bold) mice were incubated with biotinylated anti-CD3 and anti-CD4. Crosslinking was induced by addition of streptavidin and the kinetics of changes in intracellular calcium concentration as indicated by Fluo4 fluorescence was monitored by flow cytometry.

The c-Cbl RING finger mutant shows enhanced phosphorylation of Y737 and p85 association

The marked difference in Akt activation between c-Cbl A/+ and -/- thymocytes raised the possibility that this effect

could be mediated by the c-Cbl protein itself. Fyn phosphorylation of a YEAM motif at Y737 of c-Cbl (Y731 in humans) mediates an association with p85 SH2 domains, resulting in the recruitment of PI3K to the cell membrane (Hunter *et al*,

Figure 7 RING finger mutated c-Cbl protein shows increased phosphorylation of Y737 and enhanced association with p85. **(A)** Lysates from thymocytes stimulated by anti-CD3 + CD4 crosslinking were blotted with anti-p-c-Cbl (pY731), anti-c-Cbl or anti-p85 (upper panels). Lysates were also immunoprecipitated with anti-p85 antibodies and blotted with anti-phosphotyrosine, anti-c-Cbl or anti-p85 (lower panels). In this experiment, two different c-Cbl A/– mice were analyzed. **(B)** Phosphorylation of Akt is uncoupled from a requirement for coreceptor stimulation in c-Cbl A/– thymocytes. Thymocytes were stimulated with anti-CD3, anti-CD3 + CD28 or anti-CD3 + CD4 for 5 min at 37°C and lysates immunoblotted with anti-pAkt (pS473) or Akt. The results also show that CD28 crosslinking enhances pAkt levels in c-Cbl–/– thymocytes. **(C)** Phospho-Akt substrate antibody reveals a prominent substrate in A/– thymocytes with a molecular weight equivalent to GSK-3β (shown by arrow). Lysates from thymocytes stimulated with anti-CD3 + CD4 antibodies for 5 min at 37°C were immunoblotted with the pAkt substrate antibody. **(D)** GSK-3α and GSK-3β are prominent substrates in c-Cbl A/– thymocytes. Thymocytes were stimulated as indicated at 37°C and total lysates immunoblotted with pGSK-3α/β (pS21/S9) or GSK-3α/β antibodies. The results also show that CD28 crosslinking enhances phosphorylation of GSK-3 in c-Cbl–/– thymocytes. **(E)** LY294002 inhibits anti-CD3-directed death of c-Cbl A/– thymocytes. Results are from two separate experiments where unstimulated or anti-CD3-stimulated c-Cbl A/– thymocytes were incubated with or without LY294002 and thymocyte death measured by PI incorporation. The most effective inhibition of the anti-CD3 death response occurred between 10 and 25 μM while concentrations of 50 μM and greater were found to be toxic and masked the anti-CD3 effect.

1999; Arron *et al*, 2001; Grossmann *et al*, 2004). Thus, c-Cbl can positively regulate PI3K activity. Importantly, Cbl-b does not possess a p85 SH2 binding site and would therefore be unable to compensate for the loss of this function in the c-Cbl KO. A higher proportion of the mutant c-Cbl protein was phosphorylated on Y737 compared to wt c-Cbl (Figure 7A),

consistent with the anti-phosphotyrosine blot in Figure 6A. Thus, RING finger mutant thymocytes have more sites for recruiting PI3K to the cell membrane, and this could provide a mechanism for generating increased levels of phospho-Akt. Indeed, we found more mutant c-Cbl protein associated with p85 following stimulation than wt c-Cbl (Figure 7A).

Activation of Akt in RING finger mutant thymocytes is independent of coreceptor stimulation

In T cells, CD28 crosslinking is necessary for the activation of the PI3K/Akt pathway, and no detectable response is seen with CD3 stimulation alone (Appleman *et al*, 2002). However, stimulation of A/– thymocytes with either anti-CD3, anti-CD3 + CD28 or anti-CD3 + CD4 induced comparable levels of pAkt (Figure 7B). Thus in A/– thymocytes, Akt activation does not require a coreceptor signal. Interestingly, while pAkt signals in +/– thymocytes bordered on the limits of detection, crosslinking with anti-CD28 did enhance pAkt levels in c-Cbl–/– thymocytes, indicating that a CD28-mediated PI3K/Akt pathway is indeed activated in thymocytes (Figure 7B). The stronger signal in KO compared to wt thymocytes may be due to higher levels of CD28 expression on their DP thymocytes (data not shown).

To determine the functional activity of pAkt in c-Cbl A/– thymocytes, we immunoblotted lysates with a phospho-Akt substrate antibody. This revealed markedly increased phosphorylation of a protein with molecular weight equivalent to glycogen synthase kinase-3 β (GSK-3 β) (Figure 7C). GSK-3 is a well-characterized substrate of Akt, which exists in an activated unphosphorylated form in resting cells (Cross *et al*, 1995). Use of phospho-specific antibodies to GSK-3 α and GSK-3 β confirmed that activated Akt in A/– thymocytes was capable of targeting GSK-3, and that crosslinking CD3 alone was sufficient to activate this pathway (Figure 7D).

The intriguing correlation between Akt activation and cell death in response to anti-CD3 crosslinking of c-Cbl A/– thymocytes prompted us to investigate the effect of suppressing the Akt pathway with the PI3K inhibitor LY294002. Culturing c-Cbl A/– thymocytes in the presence of 10 or 25 μ M LY294002 reduced anti-CD3-directed cell death to a level equivalent to that seen in unstimulated cultures (Figure 7E). These concentrations of LY294002 were also found to markedly and selectively inhibit the activation of Akt without affecting Erk activation or c-Cbl tyrosine phosphorylation (data not shown). Thus, it is likely that the high level of Akt activation in response to anti-CD3 crosslinking is responsible for mediating *in vitro* death of c-Cbl A/– thymocytes. In future studies, it will be important to find ways of suppressing this pathway *in vivo* to determine if thymic loss can be rescued.

Discussion

Analysis of mice with a loss-of-function mutation in the c-Cbl RING finger has revealed the importance of this domain for embryonic development and thymocyte survival. This mutation has been well characterized for its ability to abolish c-Cbl's interaction with E2s without affecting associations with other signaling proteins or recruitment to activated receptors (Joazeiro *et al*, 1999; Levkowitz *et al*, 1999; Ota *et al*, 2000). By parallel analysis with the c-Cbl KO, we have identified functions that are specifically dependent on, or mediated by, the RING finger domain. Furthermore, this comparison highlights the role played by Cbl-b in embryonic development in the c-Cbl KO mouse, which may be precluded in the C379A mouse by the presence of the mutant protein.

That c-Cbl and Cbl-b can have compensatory roles in embryonic development is evident by the fact that individual KOs are developmentally normal yet the double KO is lethal

before day E10.5 (Naramura *et al*, 2002). Although embryonic death is not as severe as in the double KO, our findings suggest that in the C379A mouse the presence of the mutant c-Cbl protein can function as an effective dominant negative protein to prevent compensation by Cbl-b. The importance of this block to Cbl-b compensation became apparent when we attempted to generate homozygous c-Cbl RING finger mutant mice and found that few mice of this genotype developed beyond day 14 of embryogenesis (Figure 1C). Thus, the c-Cbl RING finger domain is functionally important for embryonic survival. Significantly, a single copy of the mutant allele on a c-Cbl null background markedly improved survival, suggesting that lowering the levels of the dominant negative protein provided a sufficient void for Cbl-b and the engagement of its RING finger.

This study also highlights the importance of the c-Cbl RING finger domain in regulating signaling events that maintain thymocyte homeostasis. Unexpectedly, in contrast to the c-Cbl KO, which has a normal, to slightly enlarged, thymus (Murphy *et al*, 1998; Naramura *et al*, 1998), we found that the RING finger mutant mouse progressively loses its thymus. Furthermore, this phenotype differs markedly from the Lck-Cre c-Cbl^{flox/flox} Cbl-b double KO mouse, which has no gross thymic changes (Naramura *et al*, 2002). These double KO mice also exhibit marked activation of peripheral T cells and the development of a fatal autoimmune disease, neither of which occurs in the c-Cbl RING finger mutant mice. The markedly different thymic phenotype to that of the c-Cbl/Cbl-b double KO indicated that the observed thymic loss in the c-Cbl RING finger knockin mouse cannot be explained by the mutant c-Cbl protein functioning as a dominant negative for Cbl-b in the thymus. Thus, the challenges from this conclusion were to discover the causal signaling events underlying this phenotype by identifying perturbations that are unique to the mutant RING finger thymus.

Analyses of thymocyte subpopulations over a range of ages showed that thymic maturation was not markedly altered in the RING finger mutant mouse, indicating that thymic loss was not caused by a developmental block. Furthermore, mixed bone marrow reconstitution experiments showed that thymic progenitors in the c-Cbl RING finger mouse are not limiting. Indeed, the RING finger mutant marrow was able to compete more successfully than either wt or c-Cbl KO marrow not only in reconstituting thymi but also in all other hematopoietic lineages (Figure 3A and B, and Supplementary Figure 2). In addition, we found that thymic deletion did not involve peripheral T-cell activation, as GK/2.43 mice, which lack peripheral T cells, were shown to lose their thymi at a rate equivalent to that of wt mice that had been repopulated with c-Cbl RING finger mutant marrow (Figure 3C). These findings prompted us to investigate whether enhanced signaling strength may be the causative event leading to thymus loss.

It is well documented that the strength and kinetics of TCR-directed signaling are key factors determining whether thymocytes are actively deleted by negative selection (Ohashi, 2003; Palmer, 2003; Starr *et al*, 2003). For this mechanism to be a plausible explanation, we would predict a marked increase in TCR signal strength in the RING finger mutant thymocytes above that of the c-Cbl KO. Our analyses however revealed many signaling identities between the two mutants, even though greater increases in CD5 and CD69 expression

were clearly evident on DP thymocytes from the RING finger mutant. Firstly, abolishing RING finger domain function produced an identical outcome to that of the c-Cbl KO in the enhancement of CD3 and TCR levels. Importantly, increased receptor levels, and downstream phosphotyrosine signaling, occur specifically in DP thymocytes and not in SP thymocytes (Supplementary Figure 4B and C). Thus, this mouse provides definitive evidence that the RING finger domain is responsible for c-Cbl's negative regulation of CD3 and TCR on DP thymocytes. This is in contrast to the normal levels of CD3 and TCR on DP thymocytes from a mouse with a loss-of-function mutation in the c-Cbl TKB domain (Thien *et al*, 2003), further highlighting that the TKB domain is not involved in the downregulation of these receptors.

We also found that ZAP-70 was not the candidate for mediating the predicted strong signal causing thymic deletion. The role of c-Cbl as a negative regulator of ZAP-70 has been extensively studied, and its activity in c-Cbl^{-/-} thymocytes is markedly enhanced and sustained (Thien *et al*, 1999). However, we found no evidence that this activity was further elevated in A^{-/-} thymocytes. When we examined a range of additional signaling proteins downstream of PTKs, we found Akt to be highly activated in c-Cbl A^{-/-} thymocytes compared to the KO. The effect of the RING finger mutation on Akt activation appears specific since the same stimulatory signals equally enhanced Erk and calcium mobilization in thymocytes from both mutant mice. One possibility for this effect is that the enhanced Akt phosphorylation, and indeed thymic loss, is a consequence of the mutant c-Cbl protein acting as a dominant negative against compensatory effects of Cbl-b. However, as mentioned above, this is unlikely since the c-Cbl/Cbl-b double KO mouse does not have a similar thymic phenotype although the presence of some residual c-Cbl protein cannot be ruled out in these mice (Naramura *et al*, 2002). Thus, the effect is more likely to be mediated by a regulatory protein upstream of Akt that is not directly involved in activating Erk or PLC γ 1. Furthermore, Akt activation was found to occur following anti-CD3 crosslinking alone, with no requirement for coreceptor ligation. Our results suggest that the candidate protein is the mutant c-Cbl protein itself that shows markedly enhanced and sustained phosphorylation of tyrosine 737, the binding site for the SH2 domains of p85. This hyperphosphorylated c-Cbl provides more sites to recruit PI3K to the cell membrane (Figure 7A). This potent gain of function is most likely through increased CD3 and Fyn levels (Figure 5A and Supplementary Figure 4), and hence an increased pool of CD3-associated Fyn that is responsible for c-Cbl phosphorylation (Deckert *et al*, 1998; Feshchenko *et al*, 1998; Hunter *et al*, 1999). Although c-Cbl is a prominent substrate of Fyn in thymocytes, little is known about the functional importance of this modification. The analysis of this mouse has therefore provided evidence that tyrosine-phosphorylated c-Cbl can play a positive role in thymocyte signaling. This mouse also highlights an unanticipated phenomenon in revealing that a multidomain protein can acquire a gain-of-function as a result of a loss-of-function mutation in another domain.

Finding a large increase in the levels of phospho-Akt following anti-CD3 crosslinking of A^{-/-} thymocytes was surprising in the light of the prominent role of Akt in promoting cell survival (Downward, 1998; Datta *et al*, 1999; Kandel and Hay, 1999). Thus, it is hard to reconcile

that thymocytes exposed to CD3 crosslinking could be susceptible to death when producing such a strong prosurvival signal. However, a recent study identifying a protein known as APE that enhances and prolongs the phosphorylation of Akt showed that its coexpression with Akt induces apoptosis in Cos-7 and HepG2 cells (Anai *et al*, 2005). Furthermore, a transgenic mouse expressing constitutively active Akt has been reported to exhibit reduced thymic cellularity (Na *et al*, 2003), although this effect is less severe and occurs in older mice compared to the RING finger mutant mouse. However, this phenotype differed from another transgenic model that found no significant effect on thymic cellularity (Jones *et al*, 2000). Like the constitutively active Akt mouse (Na *et al*, 2003), c-Cbl A^{-/-} thymocytes showed a marked enhancement in Erk activation; however, this is not increased above that of the c-Cbl KO. Importantly, however, comparison with these models may be inappropriate, as the effect we observe on Akt is clearly enhanced by antigen receptor engagement. While at present we cannot conclusively attribute thymic deletion to the markedly enhanced Akt signal in the C379A mouse, the *in vitro* effects of LY294002 are supportive of Akt involvement in a CD3-directed death signal. Furthermore, our findings offer clues for identifying the elusive point of 'signal splitting' proposed by Neilson *et al* (2004) where a high-intensity TCR signal diverts to a pathway leading to cell death. Indeed, a large gap exists in our knowledge between this point and the activation of proapoptotic pathways. In part this gap exists because many candidate proteins are involved in pre-TCR signaling, and mutations to these cause a block during DN development precluding an examination of their roles in positive and negative selection. Therefore, the c-Cbl RING finger mutant mouse represents a unique model of thymic deletion and as such has provided new opportunities for identifying TCR signaling pathways that are responsible for the negative selection of thymocytes.

Materials and methods

Generation of c-Cbl(C379A) knockin mice

A c-Cbl genomic clone from a λ UNI-ZAP 129Sv library (Murphy *et al*, 1998) was used to construct the targeting vector as shown in Figure 1A. Site-directed mutagenesis of the relevant exon created the Cys (TGT) $\rightarrow$ Ala (GCT) substitution at amino acid 379. The generation of c-Cbl(C379A) knockin mice was carried out by Ozgene Pty Ltd (Australia) and founder lines on a mixed 129Sv/J $\times$ C57BL/6 background obtained from two independently derived clones. Excision of the loxP-flanked pGKNeo cassette was induced by mating with C57BL/6 Cre-deleter transgenic males. Mice were genotyped by PCR using primers p1 (G-C404: 5'-GGACACCT CATGTGCACATCTG-3'), p2 (mG-C413rev: 5'-ATCGGCAAAAAGGA CAGCCCTGAC-3') and p3 (3'Neo: 5'-CTCGACTAGAGGATCAGCTTG-3') as indicated in Figure 1A. Mouse experiments were performed in accordance with the Animal Ethics Committee at UWA (approval 03/100/275).

Mice and bone marrow chimeras

Bone marrow cells from c-Cbl A^{-/-}, +/⁻ and -/⁻ (Murphy *et al*, 1998) mice or wt C57BL/6 CD45.1 congenic mice were left unmixed or mixed in 1:1 or 4:1 (wt:mutant) ratios. CD45.1 and C57BL/6 GK/2.43 mice (6–8 weeks old) were lethally irradiated with two doses of 5.5 Gy separated by 14 h, before injection with 2×10^6 bone marrow cells into the lateral tail vein. GK/2.43 mice are double transgenic mice for the anti-CD4 antibody (GK1.5) and the anti-CD8 antibody (2.43) that deplete CD4⁺ and CD8⁺ T cells in the periphery but do not affect thymic development (Zhan *et al*, 2000b, 2003). The antibodies are mainly produced from the pancreata under the control of the human CMV promoter (Zhan *et al*, 2000a, b). Vav-Bcl-

2 transgenic mice have been previously described (Ogilvy *et al*, 1999).

Thymocyte stimulation, immunoprecipitation and immunoblotting

Thymocytes incubated with biotinylated antibodies against CD3 (500A2), CD4 (GK1.5) or CD28 (37-51) (BD Pharmingen) were stimulated by streptavidin crosslinking on ice or at 37°C. Cells were lysed in 0.2% NP-40/60 mM *n*-β-D-glucopyranoside-containing buffer and lysates were analyzed by immunoprecipitation and immunoblotting as described previously (Thien *et al*, 1999). Lck, Fyn, PLCγ-1, MAPK and Cbl-b antibodies were purchased from Santa Cruz, ZAP-70 and c-Cbl antibodies from BD Transduction Labs and anti-p85 from UBI. Anti-ZAP-70 (R1213) and anti-phosphotyrosine (4G10) were provided by L Samelson and B Druker, respectively. Antibodies to Akt, pAkt (S473 and T308), pAkt substrate (RXRXXpS/pT), pErk(T202/Y204), SAPK/JNK and pSAPK/JNK (T183/Y185) and pGSK-3α/β(S21/9) were from Cell Signaling. pSLP-76(Y145) and pPLCγ-1(Y783) antibodies were from Biosource.

Flow cytometry

Antibody-labeled cell suspensions were collected on BD FACSCalibur or FACSCanto flow cytometers using CellQuest software (BD) and analyzed using FlowJo (Tree Star Inc.). Antibodies used were against CD3 (145-2C11), TCRβ (H57-597), CD4 (RM4-5), CD8 (53-6.7), CD5 (53-7.3), CD11b (M1/70), Ly-6G (Gr-1) (RB6-8C5), B220 (RA3-6B2), CD19 (1D3), CD25 (PC61), CD44 (IM7), CD69 (H1.2F3), TER-119, CD45.1 (A20) and CD45.2 (104) (BD). To examine DN thymocyte populations, FITC-conjugated antibodies to CD4, CD8, CD3, B220, Gr-1 and TER-119 were added, and FITC-negative cells were analyzed with anti-CD25 PE and CD44 APC. To detect intracellular levels of Fyn, Lck and phosphotyrosine, cells were fixed and permeabilized in Cytofix/Cytoperm (BD) according to the manufacturer's directions. Following incubation with antibodies,

the cells were washed in media containing 0.1% saponin and the unlabeled antibodies detected with APC-conjugated goat anti-mouse IgG (BD).

Plate-bound antibody-mediated negative selection

Tissue culture plates (96 wells) were coated overnight at 4°C with 10 μg/ml of CD3 (2C11) or CD3 + CD28 (37.51) antibodies. Thymocytes at 2×10^6 /ml were cultured in triplicate in RPMI/10% FCS for 24 h at 37°C and then harvested and stained with propidium iodide (PI) for analysis by flow cytometry. In some cultures, LY294002 (Alomone Labs) was added at 10, 25, 50 and 100 μM and thymocytes were analyzed after 18 h.

Intracellular calcium analysis

Thymocytes loaded with Fluo-4 (Molecular Probes) were labeled with biotinylated anti-CD3 and anti-CD4 antibodies followed by streptavidin crosslinking at room temperature. Changes in intracellular calcium concentrations were detected by flow cytometry and data were analyzed using CellQuest and FlowJo software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank David Izon for help with DN analysis, Jay Steer for advice with calcium response experiments, Sonja Gustin for genotyping, Simone Ross and Helen Moulder for animal care and Peter Podias and Rajin Nathan for irradiation of mice. We also thank Jerry Adams and Mark Smyth for Bcl-2 mice and Larry Samelson, Brian Druker and David Huang for antibodies. This work was supported by grants from NHMRC (Canberra) and MHRIF (Perth).

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