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A Requirement for the Rho-Family GTP Exchange Factor Vav in Positive and Negative Selection of Thymocytes

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Summary

The T cell repertoire is shaped by positive and negative selection of thymocytes that express low levels of T cell receptor (TCR) and both CD4 and CD8. TCR-mediated signals that determine these selection processes are only partly understood. Vav, a GDP-GTP exchange factor for Rho-family proteins, is tyrosine phosphorylated following TCR stimulation, suggesting that it may transduce TCR signals. We now demonstrate that mice lacking *Vav* are viable and display a profound defect in the positive selection of both class I– and class II–restricted T cells. In contrast, Vav is not essential for negative selection, though in its absence negative selection is much less effective. Vav may influence the efficiency of TCR-induced selection events by regulating the intracellular calcium flux of thymocytes.

Introduction

The T cell repertoire is shaped by the positive and negative selection of immature $CD4^+CD8^+$ double positive (DP) thymocytes that express low levels of the T cell receptor (TCR)/CD3 complex. The outcome of these selection events is determined by interactions between the TCR on DP thymocytes and peptides presented by the class I and class II molecules of the major histocompatibility complex (MHC) expressed on thymic stroma. Positive selection, which is believed to result from weak interactions between the TCR and peptide/MHC complexes, is a process that drives the development of DP thymocytes into $CD4^-CD8^+$ single positive (SP) cells capable of recognizing antigenic peptides in the context of MHC class I and $CD4^+CD8^-$ SP cells capable of recognizing peptides associated with MHC class II (Jameson et al., 1995). In contrast, the elimination of potentially selfreactive thymocytes (negative selection) is thought to be driven by strong interactions between TCR and self-MHC (Kisielow and von Boehmer, 1995). It has been postulated that these differential consequences are controlled by the avidity of the TCR/MHC interaction, which is determined by the cell surface density of peptide/MHC complexes as well as the intrinsic affinity of the TCRs and their coreceptors for their ligands (Jameson et al., 1995; Williams et al., 1997).

An unresolved question in this field has been how signals from the same TCR can result in such opposing outcomes as positive and negative selection. The TCR-mediated signals that determine these processes are only partially understood. Activation of the tyrosine kinases Lck and ZAP-70, which are coupled directly to the TCR, are essential for both positive and negative selection (Hashimoto et al., 1996; Negishi et al., 1995; Penninger et al., 1996). In contrast, activation of the Ras/extracellular signal-regulated protein kinase (ERK) mitogen-activated protein (MAP) kinase pathway is necessary, though not sufficient, for positive selection but dispensable for negative selection (Aberola-Ila et al., 1995, 1996; O'Shea et al., 1996; Swan et al., 1995; Swat et al., 1996). These results suggest that the signaling pathways leading to positive and negative selection may be distinguishable at the biochemical level.

The proto-oncogene Vav was discovered by virtue of a mutation which rendered it able to transform fibroblasts (Katzav et al., 1989). Vav contains a domain that is similar to the proto-oncogene Dbl, a guanine nucleotide exchange factor for the Rho/Rac/CDC42 family of low molecular weight Ras-like GTPases (Adams et al., 1992; Boguski et al., 1992). In addition, Vav contains multiple features, such as a pleckstrin homology domain, a single SH2, and two SH3 domains, which suggest that Vav can interact with multiple components of signal transduction pathways (Collins et al., 1997). Recent biochemical analyses as well as genetic studies in yeast have shown that Vav, when tyrosine phosphorylated, acts to promote Rac1 and other Rho-family proteins to the active GTP-bound state (Crespo et al., 1996, 1997; Han et al., 1997; Olson et al., 1996). Vav is expressed at high levels in T cells and is rapidly phosphorylated by tyrosine kinases following antigen receptor engagement in thymocytes and mature T cells (Bustelo et al., 1992; Margolis et al., 1992; Tarakhovsky et al., 1995b). Both Src family and Syk/ZAP-70 family kinases have been implicated in the tyrosine phosphorylation of Vav (Costello et al., 1996; Crespo et al., 1997; Han et al., 1997). Vav appears to regulate the transcription of genes expressed by T cells, because overexpression of Vav in Jurkat T cells enhances basal and TCR-activated transcription of the interleukin-2 (IL-2) gene and reporter constructs containing multiple NFAT binding sites (Holsinger et al., 1995; Wu et al., 1995). Using the *Rag-1*$^{-/-}$ blastocyst complementation technique, we and others have found that T cell development is impaired in the absence of Vav and that mature T cells, which lack Vav, proliferate poorly and produce little IL-2 in response to stimulation through the TCR, suggesting that Vav plays an important role in signal transduction pathways activated by the TCR (Fischer et al., 1995; Tarakhovsky et al., 1995b; Zhang et al., 1995).

To determine the role that Vav might play in thymic selection events, we established the *Vav* mutation in the mouse germline. In contrast to a previous report (Zmuidzinas et al., 1995), we show that disruption of the *Vav* gene does not result in an early embryonic lethality, but instead Vav-deficient mice are alive and healthy. The availability of such mice has enabled us to carry

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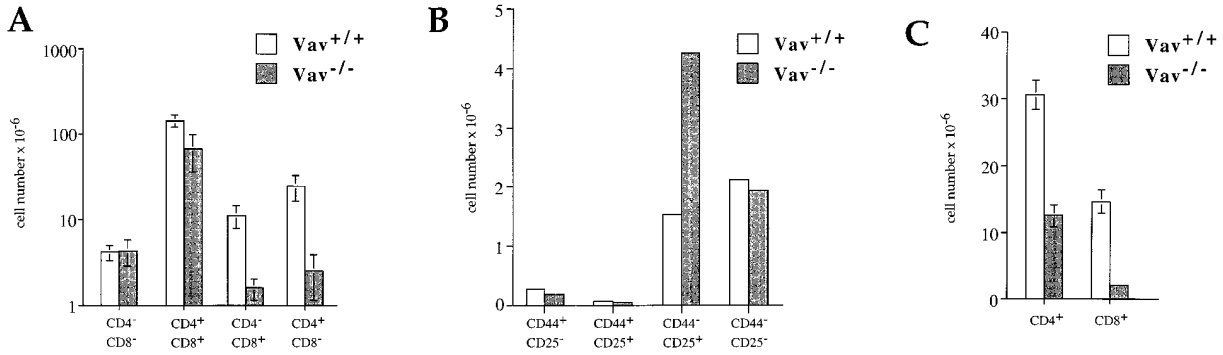

Figure 1. Impaired Thymic Development in Vav-Deficient Mice

(A) Bar graph showing mean numbers of thymocytes from five control ($Vav^{+/+}$) and five mutant ($Vav^{-/-}$) mice (129/Sv background, 6 weeks old). Error bars represent standard deviation. Numbers are shown for the least mature double negative (DN) fraction of thymocytes expressing neither CD4 nor CD8 ($CD4^-CD8^-$), with the double positive (DP) fraction expressing both ($CD4^+CD8^+$) or the most mature single positive (SP) fractions expressing either CD4 or CD8 alone ($CD4^+CD8^-$ and $CD4^-CD8^+$). Vav-deficient thymocytes show a 2-fold reduction in the number of DP and a 7- to 10-fold reduction in the number of SP cells. Note the logarithmic scale.

(B) Bar graph showing numbers of cells in the $CD4^-CD8^-$ fraction of control or mutant thymuses further subdivided according to the expression of CD44 and CD25. These subfractions are shown in their order of maturity, from the least mature $CD44^+CD25^-$ to the most mature $CD44^-CD25^-$ cells. Vav-deficient thymuses show an accumulation of cells in the $CD44^-CD25^+$ fraction.

(C) Bar graph showing mean numbers of $CD4^+$ or $CD8^+$ T cells in the spleens from two control ($Vav^{+/+}$) and four mutant ($Vav^{-/-}$) mice (129/Sv background, 6–8 weeks old). Error bars represent standard error of mean.

out a detailed study of thymic selection events in the absence of Vav. Using either class I– or class II–restricted TCR transgenes, we demonstrate that Vav is necessary for their positive selection but not for negative selection. However, an examination of superantigen-induced negative selection showed that Vav-deficient thymocytes are much less sensitive to deletion. Thus, Vav affects the efficiency of both positive and negative selection. Finally, we demonstrate that the Vav mutation greatly reduces the TCR-induced intracellular calcium flux in DP thymocytes. This may explain the reduced efficiency of thymic selection events in the Vav-deficient mice.

Results

Vav$^{-/-}$ Mice Are Viable

We have previously described the generation of embryonic stem (ES) cell lines carrying a disruption of the *Vav* gene (Tarakhovsky et al., 1995b). The mutation (*Vav*^{tm1Tyb}) was made by homologous recombination which introduced a *neo* gene into the Dbl-homologous region of Vav. These targeted ES lines were used to establish a mouse strain carrying the mutation, and interbreeding of heterozygous mutants generated homozygous mutant (*Vav*$^{-/-}$) mice at the expected frequency. *Vav*$^{-/-}$ mice are viable, healthy, and fertile, in contrast to a previous report (Zmuidzinas et al., 1995). Immunoblot analysis of thymocytes, splenic B and T cells, as well as primary mast cell cultures from the mutant mice demonstrated no detectable full-length or partial Vav protein, as expected from previous studies using *Rag-1*$^{-/-}$ chimaeras reconstituted with *Vav*$^{-/-}$ ES cells (data not shown; Tarakhovsky et al., 1995b).

Altered Thymic Development in *Vav*$^{-/-}$ Mice

Thymic cellularity of *Vav*$^{-/-}$ mice was typically half that of control mice. This can be almost entirely accounted for by a 2-fold reduction in the number of $CD4^+CD8^+$ DP thymocytes (Figure 1A, note logarithmic scale). The absolute number of $CD4^-CD8^-$ double negative (DN) cells was similar between mutant and control mice (Figure 1A). The DN compartment was further analyzed for expression of CD44 and CD25, which can be used to define four subsets of cells (Godfrey et al., 1993). Vav-deficient thymuses contained normal numbers of the earliest populations ($CD44^+CD25^-$ and $CD44^+CD25^+$) but showed a greater than 2-fold increase in the numbers of $CD44^-CD25^+$ cells (Figure 1B). A developmental block between the $CD44^-CD25^+$ and $CD44^-CD25^-$ subsets of DN thymocytes has been reported in mice mutant for Rag-1, TCRβ, and preTα, suggesting that this transition is dependent on signaling through the pre-TCR and is used to monitor for successful rearrangement of TCRβ (Fehling et al., 1995; Kisielow and von Boehmer, 1995). The existence of a similar, though not complete, block in *Vav*$^{-/-}$ mice suggests that Vav may play a role in pre-TCR signaling. Alternatively, it may be involved in the transition of DN to DP cells or the subsequent survival or expansion of the cells. The thymuses of mice heterozygous for the *Vav* mutation (*Vav*$^{+/-}$) were indistinguishable from wild-type controls in this analysis as well as in the experiments described subsequently in this paper.

Positive Selection of Both Class I– and Class II–Restricted Thymocytes Is Severely Compromised in *Vav*$^{-/-}$ Mice

In addition to the reduction in numbers of DP thymocytes, Vav-deficient mice showed a more dramatic reduction (7- to 10-fold) in the number of $CD4^+$ and $CD8^+$ SP cells, suggestive of a block in positive selection (Figures 1A and 2). A similar decrease was also seen in the numbers of $CD4^+$ and $CD8^+$ peripheral T cells in the spleen and lymph nodes of the mutant mice (Figure 1C; data not shown). A hallmark of DP thymocytes undergoing positive selection is an increase in TCR expression from intermediate to high levels and the induction of

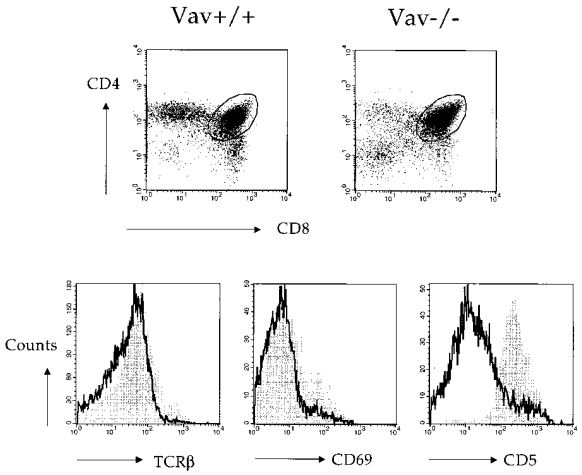

Figure 2. Thymic Development in Vav-Deficient Thymocytes

The dot plots at the top show flow cytometric analysis of a control (Vav$^{+/+}$) and Vav-deficient (Vav$^{-/-}$) thymus stained for CD4 and CD8. Expression of CD69 and CD5 in CD4$^+$CD8$^+$ DP cells (gated as shown in the dot plots) is shown in the histograms. TCRβ expression on DP cells was evaluated in a different experiment using a CD4$^+$CD8$^+$ gate equivalent to the one shown. The shaded histograms show expression in Vav$^{+/+}$ cells, and the open ones show expression in Vav$^{-/-}$ cells.

expression of CD69. Flow cytometric analysis of Vav-deficient DP thymocytes demonstrated that they contained many fewer cells that were TCR^{hi} or CD69$^+$, consistent with a failure of positive selection (Figure 2). In addition, the DP cells expressed lower levels of CD5 (Figure 2). Furthermore, the intermediate level of TCR expression found on most DP thymocytes was slightly lower in the *Vav*$^{-/-}$ mice than in controls (Figure 2, see also Figure 3A, 3B, and 3C). However, the few SP thymocytes and peripheral T cells that were found in *Vav*$^{-/-}$ mice expressed normal levels of many cell surface markers (TCR, CD4, CD8, CD69, HSA, CD44, L-selectin; data not shown).

Since Vav is expressed in multiple lineages, it was possible that this altered thymic development was a consequence of Vav deficiency in a non–T cell. For example, class II–expressing dendritic cells in the thymus are hemopoietic in origin and can influence selection events (Brocker et al., 1997). To address this issue, we reconstituted irradiated mice with a mixture of wild-type and Vav-deficient bone marrow. Flow cytometric analysis demonstrated that even in these mixed chimaeras, Vav-deficient thymocytes still did not undergo normal development, but recapitulated the large decrease in numbers of SP thymocytes and peripheral T cells (data not shown). In contrast, in the same mice, thymic development from the wild-type marrow was normal. Thus, the effect of the Vav mutation on T cell development is cell autonomous, suggesting that Vav plays a role in selection-induced signal transduction events within thymocytes.

To test the requirement for Vav in positive selection in more detail, transgenic TCRs were introduced onto the *Vav* mutant background. The F5 transgenic TCR recognizes an influenza nuclear protein peptide in the context of H-2D^b and is positively selected on the H-2^b haplotype (Mamalaki et al., 1993). F5/*Vav*$^{-/-}$ mice were further bred onto the *Rag-1*$^{-/-}$ mutation, to prevent endogenous TCR rearrangements. In these mice, all CD4$^+$CD8$^+$ and CD8$^+$ SP thymocytes expressed the transgenic TCR (Figure 3A; data not shown). The *Vav* mutation severely impaired the generation of mature CD8$^+$ cells. Additionally, in contrast to control mice, very few CD4$^+$CD8$^+$ cells in the *Vav* mutants were TCR^{hi} or CD69$^+$, consistent with a failure of positive selection (Figure 3A; data not shown). The failure to positively select CD8$^+$ cells was not unique to the F5 TCR because another MHC class I–restricted TCR, BM3.6, was similarly affected (Figure 3B). BM3.6 is an anti–H-2K^b TCR which is positively selected on an H-2^k haplotype (Sponaas et al., 1994). In BM3.6/*Rag-1*$^{-/-}$/H-2^k mice, there is exclusive selection into the CD8$^+$ SP compartment. Introduction of the *Vav* mutation onto this background abrogated the selection of these cells and once again was associated with an absence of DP thymocytes that were TCR^{hi} or CD69$^+$ (Figure 3B; data not shown).

To determine whether the positive selection of class II–restricted CD4$^+$ thymocytes also requires Vav, we crossed the *Vav* mutation with a transgenic line expressing the A1 TCR, which recognizes a peptide from the male-specific H-Y antigen presented by I-E^k and is positively selected on the H-2^k haplotype (Douek et al., 1996). Control female mice showed an exaggerated skewing toward CD4$^+$ SP thymocytes but also generated some CD8$^+$ SP cells, because the mice were *Rag-1*$^{+/+}$ and thus had rearrangements of endogenous TCR genes (Figure 3C). In contrast, in Vav-deficient female mice, few CD4$^+$ SP thymocytes were generated, and neither the DP nor CD4$^+$ SP cells expressed high levels of Vβ8.2, the Vβ gene used by the A1 transgenic TCR (Figure 3C; data not shown). Thus, positive selection of the class II–restricted A1 TCR also requires Vav.

Vav-Deficient Thymocytes Can Undergo Negative Selection

To examine the requirement for Vav in negative selection of thymocytes, we bred the Vav mutation onto a background expressing the BM3.6 TCR and H-2^b. Since BM3.6 is an alloreactive anti–H-2K^b TCR, in *Vav*$^{+/+}$ mice almost all the DP thymocytes were deleted, and thymic cellularity was reduced 100-fold (Figure 4A). Since these mice were *Rag-1*$^{+/+}$, they contained a small number of CD8$^+$ SP cells which expressed TCRs generated from endogenous TCR genes; however, these were negative for the BM3.6 clonotype (Figure 4A; data not shown). The thymuses of *Vav*$^{-/-}$ mice on this background were similarly reduced in size, contained very few DP cells, and both the DP and CD8$^+$ SP cells were negative for the BM3.6 clonotype. Therefore, Vav is not required for negative selection of this class I–restricted TCR.

Vav$^{-/-}$ Mice Have an Altered Sensitivity to Negative Selection

We also studied negative selection by endogenous superantigens. In BALB/c mice, proteins encoded by mouse mammary tumor proviruses (*Mtv* loci) are presented in association with the class II molecule I-E^d and result in

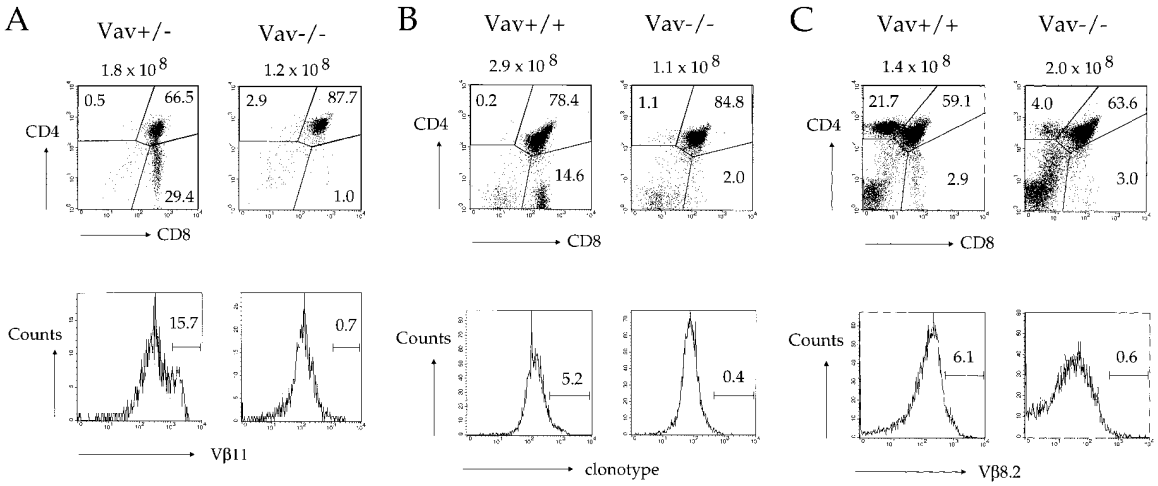

Figure 3. Positive Selection of Vav-Deficient Thymocytes

Flow cytometric analysis of control ($Vav^{+/+}$ or $Vav^{+/-}$) and mutant ($Vav^{-/-}$) mice expressing transgenic TCR. In each part, the top two dot plots show expression of CD4 and CD8 in the thymus. The total number of thymocytes is shown above each plot, and the percentage of cells falling into the DP and SP fractions is shown. The bottom two histograms show expression of transgenic TCR on the DP cells ($CD4^+CD8^+$) gated as shown in the dot plots. Markers on the histograms show the percentage of DPs that are TCR^{hi} and were placed to coincide with the expression (data not shown) of transgenic TCR in $CD8^+$ SP (A and B) and $CD4^+$ SP (C) cells in the appropriate $Vav^{+/+}$ thymuses.

(A) Control and mutant mice (6 weeks old) expressing the F5 transgenic TCR on an H-2^b background. F5 recognizes a peptide from influenza NP protein presented by H-$2D^b$ and is positively selected on H-2^b (Mamalaki et al., 1993). The mice were bred to be *Rag-1*$^{-/-}$ in order to avoid endogenous TCR rearrangements. Expression of the transgenic TCR on DP cells was evaluated using an anti-Vβ11 antibody.

(B) Control and mutant mice (8–10 weeks old) expressing the BM3.6 transgenic TCR on an H-2^k background. BM3.6 is an anti–H-$2K^b$ TCR and is positively selected on H-2^k (Sponaas et al., 1994). The mice were bred to be *Rag-1*$^{-/-}$ in order to avoid endogenous TCR rearrangements. Expression of the transgenic TCR on DP cells was evaluated using an anti-clonotype antibody.

(C) Control and mutant mice (8–10 weeks old) expressing the A1 transgenic TCR on an H-2^k background. A1 is an anti–H-Y TCR and is positively selected on I-E^k (Douek et al., 1996). Expression of the transgenic TCR on DP cells was evaluated using an anti-Vβ8.2 antibody. Since these mice are *Rag-1*$^{+/+}$, there are endogenous TCR rearrangements which result in the maturation of some Vβ8.2-expressing thymocytes (both $CD4^+$ and $CD8^+$ SP), probably as a result of pairing with endogenous TCRα chains.

the deletion of $CD4^+$ and $CD8^+$ SP cells expressing Vβ3, 5, and 11 (Simpson et al., 1993). Flow cytometric analysis of *Vav*$^{-/-}$ mice that had been backcrossed onto a BALB/c background demonstrated that in comparison with B10 mice, which do not undergo *Mtv*-mediated deletion, the Vav mutants had specifically deleted $CD4^+$ SP thymocytes expressing Vβ3, 5, and 11 (Figure 4B). In contrast, Vβ8-expressing SP cells, which do not bind the *Mtv* superantigens present in BALB/c, were unaffected. However, comparison of the *Vav*$^{-/-}$(BALB/c) with wild-type BALB/c mice demonstrated that the extent of deletion was not as great in the mutant mice. Thus, although negative selection by *Mtv* superantigens can occur in *Vav*$^{-/-}$ mice, it appears to be less efficient.

To examine the altered sensitivity of Vav-deficient thymocytes to negative selection in more detail, we analyzed their deletion by Staphylococcal enterotoxin B (SEB), which binds to class II molecules and to Vβ3, 7, 8, and 17 (Marrack and Kappler, 1990). This binding leads to the selective elimination of SP thymocytes expressing these particular Vβs. SEB-mediated negative selection is a "late" acting event and does not cause Vβ-specific deletion of DP thymocytes (data not shown). Vav-deficient and control mice were injected with different amounts of SEB and were monitored for the selective disappearance of SP cells expressing Vβ8. This allowed us to determine the dose response of SEB-driven negative selection in both genotypes. Vav-deficient mice were clearly less susceptible to deletion by SEB, since injections of 0.3 μg SEB induced half-maximal deletion in control mice, whereas ten times more SEB was required to induce a similar level of deletion in the mutant mice (Figure 4C). Thus, although not absolutely required for negative selection, Vav certainly affects the efficiency of the process.

Altered Calcium Flux in *Vav*$^{-/-}$ Thymocytes

One of the hallmarks of TCR-induced signaling is the mobilization of intracellular calcium (Ca^{2+}), and this can be demonstrated in mature T cells as well as both SP and DP thymocytes (Havran et al., 1987; Helman-Finkel et al., 1987). Furthermore, such calcium fluxes have been implicated in both positive and negative selection in the thymus (Helman-Finkel et al., 1989; Nakayama et al., 1992; Takahama and Nakauchi, 1996; Vasquez et al., 1994). To examine TCR-induced calcium flux in *Vav*$^{-/-}$ thymocytes, the cells were preloaded with the calcium-sensitive dye Indo-1, and flow cytometry was used to monitor intracellular calcium concentration following cross-linking of the TCR/CD3 complex. These studies clearly showed that calcium fluxes were greatly reduced in the Vav-deficient thymocytes, though they were not completely absent (Figure 5). In control DP and $CD4^+$ SP thymocytes, calcium fluxes were readily detectable at the lowest dose of cross-linking antibody (10 μg/ml). In contrast, in *Vav*$^{-/-}$ DP thymocytes, only at the highest dose of cross-linking antibody (80 μg/ml) was there a detectable flux. Vav-deficient $CD4^+$ SP cells were able

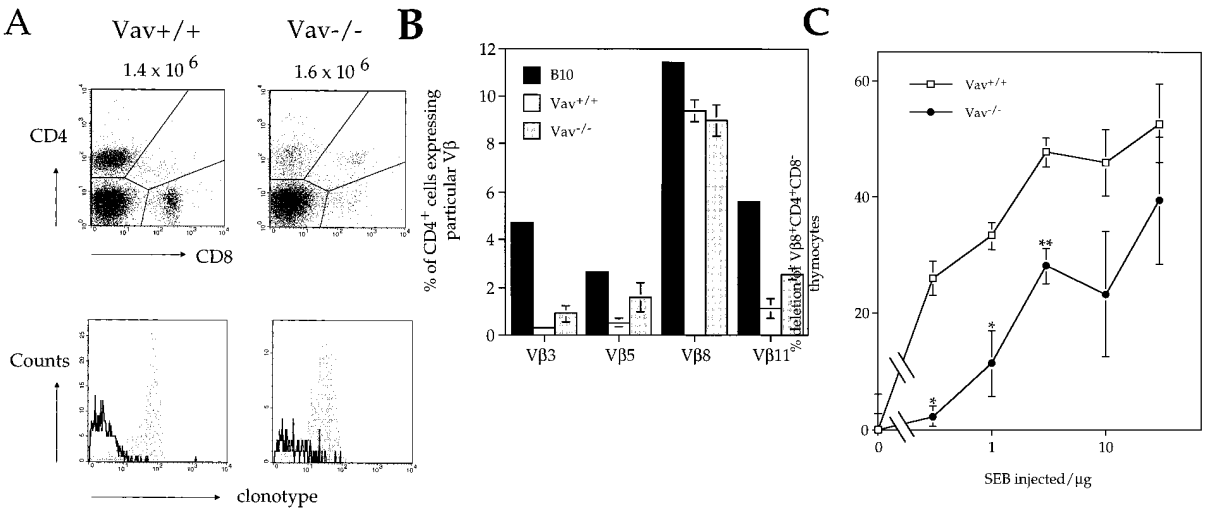

Figure 4. Negative Selection of Vav-Deficient Thymocytes

(A) Flow cytometric analysis of thymuses from control (Vav$^{+/+}$) and mutant (Vav$^{-/-}$) mice (8–10 weeks old) expressing the BM3.6 transgenic TCR on a deleting H-2^b background. These mice are *Rag-1*$^{+/+}$, so some endogenous TCR genes are expressed. The total number of thymocytes is shown above each plot. Note the very small size of the thymus (compare with Figure 3B) and the virtual absence of DP cells. The histograms show the expression of transgenic TCR in CD8$^+$ SP cells from these deleting (H-2^b) thymuses (open) and from nondeleting (H-2^k) thymuses (shaded).

(B) *Mtv* superantigen deletion of CD4$^+$ lymph node T cells expressing particular Vβ genes. The bar graph represents the mean percentage of CD4$^+$ T cells expressing Vβ3, 5, 8, or 11 in lymph nodes taken from three *Vav*$^{-/-}$ mice backcrossed onto BALB/c (Vav$^{-/-}$) and from three *Vav*$^{+/+}$ littermates (Vav$^{+/+}$). Error bars show standard error of mean. Percentages of CD4$^+$ T cells expressing the same Vβ genes in an age-matched C57Bl/10 (B10) mouse are shown for comparison. B10 mice do not express I-E and are thus unresponsive to Mtv superantigens (Simpson et al., 1993).

(C) Staphylococcal enterotoxin B (SEB)-induced deletion of Vβ8$^+$CD4$^+$ SP thymocytes. Vav-deficient (Vav$^{-/-}$) or control (Vav$^{+/+}$) mice which had been backcrossed to B10. BR were injected three times with the dose of SEB shown every other day. Mice were analyzed the day after the third injection. Graph shows the percentage deletion of Vβ8$^+$CD4$^+$CD8$^-$ thymocytes in two independent experiments; error bars represent standard error of mean. Three to eight mice were analyzed at each point. In mice injected with PBS (0 μg SEB), 25.8% (Vav$^{+/+}$) or 25.4% (Vav$^{-/-}$) of the CD4$^+$CD8$^-$ SP thymocytes were Vβ8$^+$. The percentage of Vβ6$^+$CD4$^+$CD8$^-$ thymocytes was not affected by the dose of SEB injected (data not shown). Statistical significance was evaluated using a Student's *t*-test, and significant differences between control and mutant mice are shown on the graph where $p < 0.01$(*) or $p < 0.001$(**).

to respond across the whole dose range (10–80 μg/ml) but in all cases gave a much reduced calcium flux. This diminished flux cannot be attributed to altered TCR levels, since these are normal in *Vav*$^{-/-}$ CD4$^+$ SP cells, but more likely reflects a direct role for Vav in coupling TCR activation with calcium mobilization.

Discussion

We have established a *Vav* mutation in the mouse germline and find the mice to be viable, healthy, and fertile. Furthermore, by immunoblot analysis, we can find no detectable full-length or partial Vav protein. The apparent embryonic lethality reported previously is most likely the result of a partial duplication of the targeted *Vav* locus in the ES cell line used to obtain germline transmission. Such an interpretation is supported by the unequal intensity of hybridizing fragments in Southern analysis of the clone used to obtain germline transmission (see Figure 1C in Zmuidzinas et al., 1995). Furthermore, the mutation appears to be inherited in a non-Mendelian fashion with the ratio of *Vav*$^{+/-}$ to *Vav*$^{+/+}$ mice generated from intercrosses of heterozygous mutants being about 3:1 rather than the 2:1 expected for a lethal mutation (see Table 1 in Zmuidzinas et al., 1995). Given the large number of animals analyzed, the deviation from the expected ratio is highly significant ($p < 0.002$). Taken together, these data suggest that mice homozygous for the targeted allele were incorrectly scored as heterozygotes because a partial duplication on the targeted allele had resulted in a *Vav* allele containing both the wild-type and mutant diagnostic DNA fragments.

The availability of viable mutant mice has allowed us to examine a number of aspects of thymic development that could not easily be studied using the *Rag*$^{-/-}$ blastocyst complementation approach we and others have used previously (Fischer et al., 1995; Tarakhovsky et al., 1995b; Zhang et al., 1995). It is well established that in developing thymocytes, successful rearrangement of the TCRβ chain is required for the transition of DN CD4$^-$CD8$^-$ cells to DP CD4$^+$CD8$^+$ and their subsequent proliferative expansion (Kisielow and von Boehmer, 1995; Malissen and Malissen, 1996). This transition is believed to be controlled by a signal from the pre-TCR, since disruption of the genes for preTα and CD3ε also cause a block at this developmental checkpoint (Fehling et al., 1995; Malissen et al., 1995). Characteristically, this developmental arrest in the DN population occurs between a subset of cells that are CD25$^+$CD44$^-$ and CD25$^-$CD44$^-$. Our results now show that Vav may also play a role in this transition, albeit a partial one, because

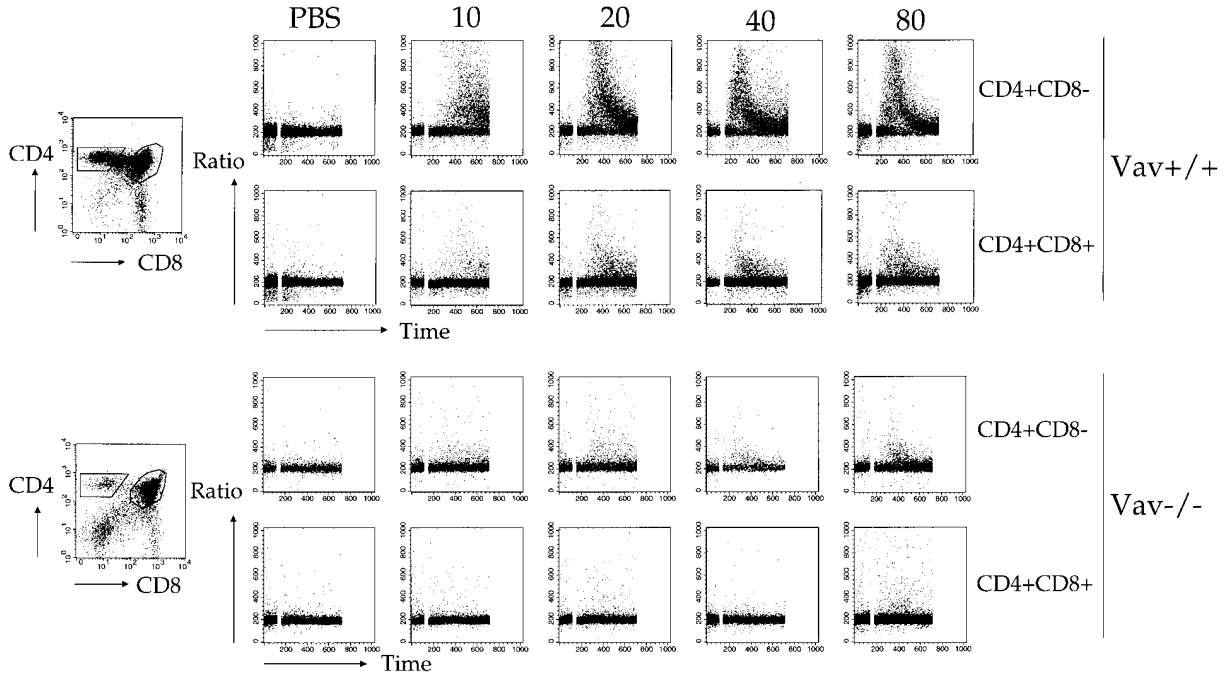

Figure 5. Intracellular Calcium Flux Is Reduced in Vav-Deficient Thymocytes

Flow cytometric analysis showing CD4 and CD8 staining of Vav-deficient ($Vav^{-/-}$) or control ($Vav^{+/+}$) thymocytes preloaded with Indo-1 and stained with anti-CD3. Intracellular [Ca^{2+}] in either the $CD4^+CD8^-$ SP or $CD4^+CD8^+$ DP thymocytes (gated as shown in the CD4/CD8 plots) is shown as a ratio of Indo-1 violet/blue fluorescence (Ratio, calcium bound dye:calcium free dye) versus time. Each channel on the time axis corresponds to 500 msec. Cells were stimulated as indicated either with PBS or 10, 20, 40, or 80 μg/ml goat anti-hamster antibody to crosslink the anti-CD3 at the time indicated by the break in the calcium trace (usually 60 sec after start of data collection). In the experiment shown, at 80 μg/ml of crosslinking antibody, 7.4% of the control and 1.5% of the mutant DP cells responded, whereas treatment with PBS resulted in a response in 0.4% and 0.3% of the control and mutant cells. Responding cells were enumerated 80–200 sec after the addition of the stimulus and were taken as responding if their calcium ratio exceeded 300.

we observed a decrease in the number of DP thymocytes and a partial block between the $CD25^+CD44^-$ and $CD25^-CD44^-$ subsets of DN thymocytes (Figure 1B). Interestingly, mice deficient for CD45, another molecule implicated in TCR signaling, show a similar reduction in the number of DP thymocytes and an accumulation of $CD44^-CD25^+$ cells in the DN compartment (Byth et al., 1996; P.J.M., M.T., and V.L.J.T., unpublished data).

The positive and negative selection of $CD4^+CD8^+$ DP thymocytes is governed by interactions between the TCR and MHC/peptide expressed on thymic stroma. Both processes require the transduction of signals from the TCR as well as signals from costimulatory molecules (Jameson et al., 1995; Kisielow and von Boehmer, 1995). Using *Rag-1*$^{-/-}$ chimaeras complemented with *Vav*$^{-/-}$ ES cells, we and others demonstrated a reduction in the number of SP thymocytes, suggesting that Vav may play a role in positive selection (Fischer et al., 1995; Tarakhovsky et al., 1995b; Zhang et al., 1995). The viable Vav-deficient mice have allowed us to examine Vav's role in both positive and negative selection in detail. We have shown that DP thymocytes in *Vav*$^{-/-}$ mice have reduced numbers of cells that are TCR^{hi} or $CD69^+$, which have been shown to be markers of DP cells that are being positively selected (Figure 2; Ohashi et al., 1990; Swat et al., 1993; Yamashita et al., 1993). This deficiency in positive selection could be due to the effect of Vav in thymocytes or in other cells, such as thymic stroma. Since in the mixed radiation chimaeras the same block in positive selection was observed in Vav-deficient thymocytes, it is most likely that the Vav mutation is having its effect within thymocytes themselves, rather than in some accessory cell. The lower levels of CD5 expression on *Vav*$^{-/-}$ DP thymocytes (Figure 2) may be a consequence of an impaired pre-TCR signal in the Vav mutants, since in wild-type mice the expression of CD5 is induced as cells transit from the DN to DP compartment. Since CD5 has been proposed to be a negative regulator of TCR signaling (Tarakhovsky et al., 1995a), the lower CD5 expression on *Vav*$^{-/-}$ DP cells may compensate in part for the lower efficiency of TCR signaling, though clearly it does not do so completely.

By crossing the Vav mutation with three TCR transgenic mouse strains, we have shown that Vav is required for the positive selection of all three receptors (Figures 3A, 3B, and 3C). This is evidenced by the lack of SP cells expressing the transgenic TCR. In the case of the A1 TCR, since the mice are *Rag-1*$^{+/+}$, some SP cells develop by virtue of expressing endogenous TCR genes. A similar phenomenon is also seen in nontransgenic *Vav*$^{-/-}$ mice, where SP thymocytes are present and peripheral T cells accumulate, albeit in reduced numbers (Figure 1A). These results suggest that the efficiency of positive selection has changed in *Vav*$^{-/-}$ mice, with positive selection being either more or less efficient, such that the signal generated by binding of the trans-

genic TCRs to their positively selecting ligand is now either too weak, causing the thymocytes to die by neglect, or too strong, resulting in their negative selection. For reasons outlined below, we think it more likely that the signals governing positive selection in Vav mutants are weaker. If this is the case, SP thymocytes and peripheral T cells that appear in the nontransgenic *Vav*$^{-/-}$ mice may be expressing TCRs with a higher affinity/avidity for positively selecting MHC ligands. This would result in a signal strong enough to overcome the Vav mutation. In a wild-type mouse, such TCRs may bind to the MHC ligand too strongly and thus be negatively selected. To test this hypothesis, rearranged TCR genes from *Vav*$^{-/-}$ T cells could be reintroduced into a *Vav*$^{+/+}$ background, whereupon they should delete.

Analysis of negative selection in *Vav*$^{-/-}$/BM3.6 mice showed that deletion of autoreactive thymocytes occurred efficiently in the absence of Vav (Figure 4A). In the case of this receptor, it is likely that the deleting ligand, the class I molecule H-2K^{b} is expressed at high levels in the thymus. However, a role for Vav in negative selection was suggested by the observation that *Mtv* superantigen-induced deletion of thymocytes expressing particular Vβ genes was less effective in *Vav*$^{-/-}$ mice (Figure 4B). To analyze this further, we injected varying doses of the superantigen SEB into mutant and control mice and measured specific Vβ deletion (Figure 4C). These studies clearly demonstrated that *Vav*$^{-/-}$ mice have a reduced efficiency of negative selection. Since negative selection can still be induced in the mutant thymuses by higher doses of TCR ligand, it is likely that the effect of the Vav mutation has been to make TCR signaling less efficient. Taken together, our results demonstrate that in the absence of Vav, both positive and negative selection of thymocytes is less efficient, probably because the signals from the TCR in *Vav*$^{-/-}$ thymocytes are weaker. One prediction of this hypothesis would be that low doses of ligands (e.g., SEB), which normally induce negative selection in wild-type thymocytes, might cause positive selection in Vav mutants. Such an outcome was never observed in our experiments.

What role does Vav play in the biochemical pathways leading from the TCR which result in positive or negative selection? Many different signaling pathways have been implicated in the positive and negative selection of thymocytes. Inactivation of the ZAP-70 and Lck tyrosine kinases profoundly affects both processes, as do mutations in CD45, a tyrosine phosphatase implicated in the activation of Lck (Byth et al., 1996; Wallace et al., 1997; P.J.M., M.T., and V.L.J.T., unpublished data). A number of studies point to the involvement of intracellular calcium fluxes and the activation of PKC in both processes (Kane and Hedrick, 1996; Takahama and Nakauchi, 1996; Vasquez et al., 1994; Wang et al., 1995). In contrast, the Ras/Raf/MEK/ERK pathway appears to be important for positive but not negative selection, whereas CD40 ligand and Jak3 have each been shown to play a role in negative but not positive selection (Aberola-Ila et al., 1995, 1996; Foy et al., 1995; O'Shea et al., 1996; Saijo et al., 1997; Swan et al., 1995; Swat et al., 1996).

In which, if any, of these pathways does Vav play a role? We have shown that DP and SP thymocytes from Vav mutants show a much reduced calcium (Ca^{2+}) flux in response to TCR stimulation compared with wild-type cells, suggesting that a defect in this pathway may account for the reduced efficiency of positive and negative signaling. An earlier report claimed that Vav-deficient DP cells failed to flux intracellular calcium (Fischer et al., 1995). We now show that although at lower doses of TCR stimulation no calcium flux can be seen in the mutant cells, at high doses some calcium flux is evident, and thus the Vav mutation has reduced the efficiency with which TCR stimulation leads to an intracellular calcium flux. A role for calcium fluxes in thymocyte selection has been suggested by a number of studies. A combination of calcium ionophore and phorbol esters can replace the TCR signals that lead to positive selection of CD4^{+} T cells (Takahama and Nakauchi, 1996), while blocking calcium fluxes with an intracellular calcium chelator resulted in a complete failure of negative selection (Vasquez et al., 1994). In contrast, blocking extracellular influx but not intracellular release of calcium abrogated negative selection in response to weak but not strong ligands (Kane and Hedrick, 1996). Furthermore, inhibition of the calcium-activated phosphatase calcineurin with FK506 or Cyclosporin A blocks positive selection and negative selection by weakly deleting ligands (Kane and Hedrick, 1996; Wang et al., 1995). These observations are very much in line with the effects of the Vav mutation: an inhibition of positive selection and of negative selection to lower doses of deleting ligand.

How does Vav modulate the TCR-induced intracellular calcium flux? Analysis in vitro as well as in mammalian and yeast cells has demonstrated that Vav is a GTP/GDP exchange factor for Rac1, RhoA, and CDC42Hs, all members of the Rho family of small GTPases (Crespo et al., 1996, 1997; Han et al., 1997; Olson et al., 1996). This activity was shown to be dependent on tyrosine phosphorylation, suggesting that the TCR-activated tyrosine phosphorylation of Vav could lead to the activation of Rho-family proteins. This family of GTPases has been implicated in many processes, including the activation of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) leading to increased production of phosphatidylinositol 4,5 bisphosphate (PIP_2; Chong et al., 1994; Hartwig et al., 1995; Ren et al., 1996; Tolias et al., 1995). PIP_2 in turn is the substrate for phospholipase C (PLC), the activation of which, following TCR stimulation, leads to the production of inositol 3,4,5 trisphosphate (IP_3) and subsequent intracellular calcium release (Berridge, 1993). Thus a failure to activate PIP5K in Vav-deficient thymocytes would lead to a shortage of PIP_2, decreased production of the second messenger IP_3, and hence a diminished calcium flux. Though we have no evidence for a failure to activate PIP5K in Vav-deficient thymocytes, such a hypothesis is given support by studies of CD19 signaling in Vav-deficient B cells. CD19 is a transmembrane signaling molecule which when engaged becomes tyrosine phosphorylated on its intracellular domain, to which Vav binds and in turn also becomes phosphorylated (Weng et al., 1994). In B cells taken from *Vav*$^{-/-}$ mice, both the CD19-induced PIP5K activation and intracellular Ca^{2+} flux are greatly reduced, suggesting that a similar mechanism may explain the

defective TCR-induced calcium flux in Vav-deficient thymocytes (L. O'Rourke, R. Tooze, M.T., D.M. Sandoval, R.H. Carter, V.L.J.T., and D.T. Fearon, unpublished data).

Rho-family GTPases have also been implicated in the regulation of other signaling pathways. For example, they have been shown to lead to the activation of the JNK signaling pathway (Coso et al., 1995; Lamarche et al., 1996; Minden et al., 1995; Olson et al., 1995). Furthermore, Vav can transduce a signal that leads to the activation of the JNK pathway in a Rac1-dependent fashion (Crespo et al., 1996; Teramoto et al., 1997). In T cells it has been proposed that the JNK pathway may be activated in part by signals from the TCR, and the same pathway may also apply in thymocytes (Su et al., 1994). Thus, a possible role for Vav in thymocyte signaling may be to transduce a signal from the TCR via Rac1 to the activation of JNK. Such a possibility could be investigated by studying JNK activation in *Vav*$^{-/-}$ DP thymocytes.

The effect of the Vav mutation on positive and negative selection, on the DN to DP transition, as well as on TCR-induced calcium flux was to reduce the efficiency of these processes, but not to block them completely. This could be explained if the mechanism of Vav's action was through the activation of PIP5K, since a Vav mutation would eliminate only the increase in PIP_2 above constitutive levels; there would still be some PIP_2 left as a substrate for PLC, and a reduced calcium flux could still occur. Alternatively, the incomplete block in *Vav*$^{-/-}$ mice may be due to compensation by Vav2, a recently described homolog of Vav, which is also expressed in the thymus (Schuebel et al., 1996). This possibility could be addressed by generating mice lacking both Vav and Vav2.

In conclusion, we have shown that *Vav*$^{-/-}$ mice are viable and fertile but display impaired positive and negative selection of both class I– and class II–restricted thymocytes. This defect may be a consequence of the decreased TCR-induced intracellular calcium flux in Vav-deficient thymocytes.

Experimental Procedures

Mice

All strains were bred at the National Institute for Medical Research. D3 ES cells were targeted with pPNTVavNeo as described previously (Tarakhovsky et al., 1995b). Chimaeric mice were generated by injecting targeted ES cells into blastocysts from C57Bl/6 mice using standard procedures. The *Vav* mutation was established in the germline by breeding chimaeras to 129/Sv mice and typing progeny by Southern blotting using standard procedures. Heterozygous *Vav* mutants were backcrossed to BALB/c and B10.BR strains for six generations before intercrossing to generate homozygous *Vav* mutants. T cell receptor transgenic mice have been described previously: BM3.6 (Sponaas et al., 1994), F5 (Mamalaki et al., 1993), and A1(m-2) (Douek et al., 1996). *Rag-1*$^{-/-}$ mice were kindly provided by D. Baltimore (Spanopoulou et al., 1994). All mice were analyzed between 4 and 12 weeks of age.

Staining of Cells with Fluorescent Antibodies

Staining with the indicated combinations of monoclonal antibodies was performed in the presence of 10% heat-inactivated normal rat serum, and fluorescence was acquired and analyzed with Cellquest software (Becton-Dickinson). Dead cells were excluded on the basis of low forward light scatter, and only live cells falling within the lymphocyte scatter gate are shown. For enumeration of early T cell subsets, thymocytes were stained with anti–CD3-biotin, anti–CD4-biotin, anti–CD8-Allophycocyanin (APC), anti–B220-APC, anti–Gr1-biotin, anti–Mac-1-Biotin, anti–CD44-phycoerythrin (PE), and anti–CD25-fluorescein isothiocyanate (FITC). Biotin-conjugated antibodies were revealed with streptavidin-APC. To distinguish early thymocyte subsets, the CD25 and CD44 expression of cells failing to stain with APC was plotted. When staining with Vβ-specific antibodies, anti-FcγRII (clone 2.4G2) was included at 20 μg/ml to reduce nonspecific binding. Antibodies to CD3 (145-2C11), CD8 (YTS 169.4), CD25 (7D4), TCRβ (H57-597), pan-Vβ8 (F23.1), Vβ8.2 (F23.2), Vβ11 (KT 11.5), and BM3.6 TCR clonotype (Ti98) were purified from tissue culture supernatants and conjugated to FITC or biotin by standard methods. All other antibodies were purchased from Pharmingen (San Diego, CA), except anti–CD4-PE (Boehringer Mannheim, Mannheim, Germany). Biotinylated antibodies were revealed with streptavidin-PE (Biogenesis Ltd., Poole, U.K.), streptavidin-RED613 or streptavidin-RED670 (GIBCO-BRL, Grand Island, NY), or streptavidin-APC (Pharmingen).

Staphylococcus Enterotoxin-B Mediated Thymocyte Deletion

Eight to ten-week-old mice were injected intraperitoneally three times on alternate days with the indicated doses of SEB (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS). On the day following the final injection, thymi were removed and stained for CD4, CD8, and Vβ8, as well as CD4, CD8, and Vβ6 (which is not deleted by SEB [White et al., 1989]). Deletion was assessed by gating on the $CD4^+$ single positives and determining the proportion of $V\beta8^+$ cells. Percentage deletion was calculated as $P_n = 100(p_0 - p_n)/p_0$, where P_n is the percentage deletion after injection of n μg of SEB, and p_0 and p_n represent the percentage of $CD4^+$ SP cells that were $V\beta8^+$ after injection with 0 μg (i.e., PBS) or n μg of SEB.

Intracellular Calcium Analysis

Thymocytes were incubated with Indo-1 acetoxy-methyl ester (Indo-1; 1 μM; Calbiochem, La Jolla, CA) for 45 min at 37°C in RPMI1640, 1% bovine serum albumin, washed, and stained with anti–CD8-FITC, anti–CD4-PE, and anti-CD3. Four-color flow cytometric analysis was performed on a FACSVantage dual-laser flow cytometer (Becton-Dickinson). FITC and PE were excited by an argon ion laser (488 nm, 100 mW) and Indo-1 by a UV argon ion laser (320 nm, 50 mW). Indo-1 emission was detected using 405/40 nm (violet) and 495/20 nm (blue) bandpass filters. To determine the relative intracellular calcium concentration, cells were warmed to 37°C, analyzed for 30–45 sec to establish baseline calcium levels, and CD3 was cross-linked by the addition of the indicated concentrations of goat anti–hamster-IgG (Jackson Immunoresearch). Acquisition was continued in real time for up to 6 min.

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