Published in final edited form as:

Sci Signal. ; 2(101): ra83. doi:10.1126/scisignal.2000420.

Function of the Nucleotide Exchange Activity of Vav1 in T cell Development and Activation*

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Abstract

The guanine nucleotide exchange factor (GEF) Vav1 is essential for transducing T cell antigen receptor (TCR) signals and therefore plays a critical role in the development and activation of T cells. It has been presumed that the GEF activity of Vav1 is important for its function; however, there has been no direct demonstration of this. Here, we generated mice expressing enzymatically inactive, but normally folded, Vav1 protein. Analysis of these mice showed that the GEF activity of Vav1 was necessary for the selection of thymocytes and for the optimal activation of T cells, including signal transduction to Rac1, Akt, and integrins. In contrast, the GEF activity of Vav1 was not required for TCR-induced calcium flux, activation of extracellular signal-regulated kinase (ERK) and protein kinase D1 (PKD1), and cell polarization. Thus, in T cells, the GEF activity of Vav1 is essential for some, but not all, of its functions.

Introduction

The Vav family of guanine nucleotide exchange factors (GEFs) catalyze nucleotide exchange on Rho family guanosine triphosphatases (GTPases), thereby leading to their activation (1, 2). Although the Rac GTPases appear to be their preferred substrates, Vav proteins will also catalyze nucleotide exchange on RhoA, RhoG, and Cdc42 (1, 3). Vav proteins play a critical role in various biological processes, including angiogenesis, axon guidance, the functions of macrophages, neutrophils, and osteoclasts, and in the development and function of lymphocytes (4-8). Vav1 is ectopically expressed in ~ 50% of pancreatic adenocarcinomas (9), which correlates with poorer survival, suggesting that inhibitors of the activity of Vav1 may have therapeutic potential (10).

Studies of Vav1-deficient mice have shown that in the absence of Vav1, the development of T cells is partially blocked at the pre-T cell antigen receptor (TCR) checkpoint in the thymus and is strongly blocked in both positive and negative selection of T cells (11-15). Furthermore, TCR-induced activation and proliferation is greatly reduced in Vav1-deficient T cells, as are multiple TCR-induced signaling pathways, including Ca²⁺ flux and the activation of extracellular signal-regulated kinase (ERK), protein kinase D1 (PKD1), the serine-threonine kinase Akt, and the transcription factors nuclear factor of activated T cells (NFAT) and nuclear factor κ B (NF- κ B) (16-20). Vav1 is also required to transduce TCR signals that lead to cytoskeletal remodeling, integrin activation, and cell polarization (17, 18,

*This manuscript has been accepted for publication in Science Signaling. This version has not undergone final editing. Please refer to the complete version of record at <http://www.sciencesignaling.org/>. The manuscript may not be reproduced or used in any manner that does not fall within the fair use provisions of the Copyright Act without the prior, written permission of AAAS.

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21-23). Despite these studies, it remains unclear what role, if any, the GEF activity of Vav1 plays in pathways known to require Vav1. Indeed, the presence of one Src homology 2 (SH2) domain and two SH3 domains in Vav1 and the identification of several interacting proteins have led to the suggestion that Vav1 may have GEF-independent functions (24); however, there is no conclusive evidence for or against this hypothesis.

To address the role of the GEF activity of Vav1 in its function, we generated mice that expressed a mutant Vav1 protein that lacked GEF activity, but which was nonetheless normally folded and hence retained any potential GEF-independent functions. We showed that this mutation affected the development and activation of T cells, thus demonstrating a critical role for the GEF activity of Vav1. However, the mutation affected only a subset of Vav1-dependent developmental processes and signaling pathways, showing unequivocally that Vav1 also has GEF-independent functions.

Results

Generation of mice expressing a GEF-inactive Vav1

To establish the functional importance of the GEF activity of Vav1 in T cell development and signaling, we aimed to generate mice expressing a mutant Vav1, which, although enzymatically inactive, retained normal folding of all eight domains and would thus preserve any potential non-GEF functions. The enzymatic activity of Vav1 resides in the Dbl homology (DH) domain, a conserved domain found in most GEFs specific for Rho-family GTPases. On the basis of the structure of the DH domain of the GEF Tiam1 in complex with Rac1 (25), we designed a mutation of Vav1 that would eliminate GEF activity but would not affect folding of the domain. In the Tiam1-Rac1 complex, Leu¹¹⁹⁴ and Lys¹¹⁹⁵ of Tiam1 make key contacts with the switch II region of Rac1 and thereby play a critical role in nucleotide exchange. Mutation of these residues to alanine reduces the exchange activity of Tiam1. Comparison with the amino acid sequence of Vav1 showed that these two residues are conserved and correspond to Leu³³⁴ and Lys³³⁵ in Vav1, occupying the same position in the structure of the DH domain of Vav1 (26). Thus, we mutated these two residues to alanine to generate Vav1(Leu³³⁴→Ala,Lys³³⁵→Ala), henceforth termed Vav1^{AA}. We and others have reported the structure of the DH, pleckstrin homology (PH), and C1 domains of Vav1 in complex with Rac1, and, as predicted, the side chains of Leu³³⁴ and Lys³³⁵ lie within the interface between the DH domain of Vav1 and Rac1 and make key contacts between the two (Fig. 1A) (3, 27).

To verify that the mutations eliminated enzymatic activity but left the domain structure and stability intact, we expressed and purified wild-type and mutant Vav1 DH domains from *Escherichia coli*. Circular dichroism spectroscopy showed that the mutant DH^{AA} domain had a secondary structure identical to that of the wild-type domain, and measurements of thermal stability showed that the mutant domain was as stable as the wild-type domain (Fig. 1B). In contrast, the mutant domain had no detectable exchange activity on Rac1 (Fig. 1C). We introduced the two amino acid mutation into the germline of mice by gene-targeting techniques, resulting in generation of mice carrying the *Vav1*^{AA} allele (Fig. 1, D to F). We chose to use gene targeting to faithfully replicate the spatial and temporal patterns of *Vav1* expression. The abundance of Vav1 protein in CD4⁺ T cells from *Vav1*^{AA/AA} mice was 67 to 84% of that in wild-type T cells, depending on the protein to which the abundance of Vav1 was normalized (Fig. 2A). The reason for this slight reduction is not known, but may be due to slightly altered patterns of gene expression in the mutant T cells. To control for this mild reduction in the abundance of Vav1, we used *Vav1*^{+/-} mice as well as wild-type mice throughout this study as a comparison. The abundance of Vav1 in *Vav1*^{+/-} T cells was 41 to 56% of that in wild-type T cells, which was less than was found in *Vav1*^{AA/AA} cells (Fig. 2A). Finally, we examined TCR-induced tyrosine phosphorylation of Vav1, which is

most likely catalyzed by the ζ chain-associated protein kinase of 70 kD (ZAP70). This phosphorylation occurred normally in $Vav1^{AA/AA}$ CD4⁺ T cells, confirming that the GEF-inactive Vav1 was folded correctly and could couple to its upstream signaling partners as efficiently as did wild-type Vav1 (Fig. 2B).

The $Vav1^{AA}$ mutation affects only a subset of Vav1-dependent thymic selection processes

Analysis of T cell development in $Vav1^{AA/AA}$ mice showed that, unlike in Vav1-deficient ($Vav1^{-/-}$) mice, the total number of thymocytes was not affected (Fig. 3A). The reduction in cell numbers in $Vav1^{-/-}$ mice is mainly due to defective transition of cells through the pre-TCR checkpoint, leading to a reduction in the number of CD4⁺CD8⁺ double positive (DP) thymocytes, which did not occur in $Vav1^{AA/AA}$ mice (Fig. 3B). In CD4⁺CD8⁺ double negative (DN) thymocytes, successfully rearranged TCR β protein forms part of the pre-TCR, which transduces signals that lead to their survival, proliferation, and maturation into DP cells. In $Vav1^{-/-}$ thymocytes, the defective pre-TCR transition is associated with an increased number of cells in DN thymocyte subsets 2 and 3 (DN2 and DN3), which immediately precede the pre-TCR checkpoint (Fig. 3A), as well as by the decreased abundance of CD5 on DP thymocytes (Fig. 3C) (15). In $Vav1^{AA/AA}$ mice, the abundance of CD5 was normal on DP thymocytes (Fig. 3C), and whereas the number of DN2 and DN3 cells was mildly elevated compared to that of wild-type mice, it was less affected than in $Vav1^{-/-}$ mice (Fig. 3A). We conclude that the Vav1^{AA} mutation had little or no effect on the pre-TCR checkpoint, and that the GEF function of Vav1 was not required for this transition. These results show that Vav1 has GEF-independent functions at the pre-TCR checkpoint.

DP thymocytes that have a mature TCR $\alpha\beta$ with low avidity for the complex between self-peptide and the major histocompatibility complex (MHC) undergo a process of positive selection into either CD4⁺CD8⁺ single positive (CD4SP) or CD4⁺CD8⁺ single positive (CD8SP) thymocyte subsets. Conversely, DP thymocytes that have a TCR with high avidity for self-peptide-MHC are eliminated by negative selection. The outcome of both of these selection events is critically dependent on TCR signaling, and both processes are defective in $Vav1^{-/-}$ mice, which results in a large decrease in the numbers of SP thymocytes and peripheral T cells (15). Analysis of cell numbers showed that despite having normal numbers of DP cells, $Vav1^{AA/AA}$ thymi had greatly reduced numbers of CD4SP and CD8SP cells, similar to those found in $Vav1^{-/-}$ mice (Fig. 3B). These reduced cell numbers persisted in the periphery, where the numbers of CD4⁺ and CD8⁺ T cells were greatly reduced in the spleen and lymph nodes of $Vav1^{AA/AA}$ mice compared to those in wild-type mice (Fig. 3D). This reduction was due to a decrease in the numbers of naïve (CD44^{lo}) CD4⁺ and CD8⁺ T cells. The numbers of activated or memory (CD44^{hi}) CD4⁺ and CD8⁺ T cells and those of CD4⁺CD25⁺ regulatory T cells were not significantly reduced (Fig. 3, E and F). Despite this developmental block, $Vav1^{AA/AA}$ T cells had normal amounts of TCR β on the surface (Fig. 3G). We noted that no decreases in the numbers of CD4SP and CD8SP thymocytes or in the number of peripheral T cells were seen in $Vav1^{+/-}$ mice (Fig. 3H), demonstrating that the selection defects in $Vav1^{AA/AA}$ mice were not caused by the slightly reduced abundance of Vav1 protein but were a consequence of the GEF-inactivating mutation.

To confirm these selection defects, we bred the $Vav1^{AA/AA}$ mice with transgenic strains of mice that expressed either the MHC class I-restricted F5 TCR or the MHC class II-restricted TCR7 TCR. To simplify analysis, we bred these mice with $Rag1^{-/-}$ mice to eliminate rearrangements of endogenous TCR-encoding genes and thus ensure a monoclonal TCR repertoire. Transgenic mice expressing the F5 TCR develop exclusively CD8SP thymocytes and CD8⁺ T cells (28). Deleting *Vav1* or introducing the $Vav1^{AA}$ mutation resulted in similarly large decreases in the numbers of CD8SP thymocytes and CD8⁺ T cells in the spleen and lymph nodes and in the ratio of CD8SP:DP thymocytes, a measure of the

efficiency of selection (Fig. 4A). TCR7 transgenic mice develop mainly CD4SP thymocytes and CD4⁺ peripheral T cells (29). Once again, deleting *Vav1* or introducing the *Vav1*^{AA/AA} mutations resulted in a very large decrease in the number of thymic CD4SP cells and the numbers of splenic and lymph node CD4⁺ T cells as well as in the ratio of CD4SP:DP thymocytes (Fig. 4, B and C). The TCR7 transgene does allow the development of some CD8SP thymocytes, although very few of these make it into the peripheral T cell pool. The *Vav1* mutations also did not permit development of substantial numbers of CD8⁺ T cells in the TCR7 transgenic mice. Taken together, these results demonstrate that the GEF activity of *Vav1* is essential for the positive selection of both CD4⁺ and CD8⁺ T cell lineages.

To examine negative selection, we crossed the *Vav1*^{-/-} and *Vav1*^{AA/AA} mice onto the B10.BR genetic background, in which endogenous superantigens cause the deletion of thymocytes that express Vβ5, Vβ11, and Vβ12 TCRβ variable regions, but not those that express Vβ8. We found that in *Vav1*^{AA/AA} mice, deletion of these thymocytes was less efficient than that in wild-type mice, but more efficient than in *Vav1*^{-/-} mice (Fig. 4D). These results indicate that the GEF activity of *Vav1* is required for efficient negative selection, but that GEF-independent functions of *Vav1* also contribute to this process.

The GEF activity of *Vav1* is required for efficient T cell activation and induction of gene expression

Vav1-deficient T cells show substantial defects in TCR-induced activation and proliferation. Examination of the same processes in *Vav1*^{AA/AA} CD4⁺ T cells showed that in response to suboptimal stimulation of the TCR, the GEF mutation strongly inhibited increases in cell size, commitment to proliferation, and increases in the abundance of CD4 and CD25 [the α chain of the interleukin-2 (IL-2) receptor] at the cell surface (Fig. 5A). This defect was observed whether the cells were stimulated only through the TCR or also through the costimulatory receptor CD28, and in all cases the defect was at least as severe as that seen in *Vav1*^{-/-} T cells. Use of saturating stimulation conditions showed that whereas *Vav1*^{-/-} T cells could increase the abundance of CD25 and proliferate to a similar extent as wild-type T cells if given a strong enough stimulus, *Vav1*^{AA/AA} T cells did not reach the same level of TCR-induced proliferation (Fig. 5B). Because only a mild defect in T cell proliferation was seen in *Vav1*^{+/-} T cells (Fig. 5C), we conclude that the GEF activity of *Vav1* is required for TCR-induced activation and proliferation of T cells.

An immediate consequence of T cell activation is the increased production of cytokines. Previous studies showed that *Vav1*-deficient T cells have defects in TCR-induced secretion of IL-2 (12, 14, 17, 18). Examination of this process showed that *Vav1*^{AA/AA} CD4⁺ T cells were also defective in IL-2 secretion, though they were less affected than were *Vav1*^{-/-} cells (Fig. 6A). The TCR-induced increase in the abundance of *Il2* mRNA was not affected by either deletion of *Vav1* or the GEF mutation, demonstrating that these defects in IL-2 secretion are due to a role for *Vav1* and its GEF activity in post-transcriptional regulation of IL-2 (Fig. 6B). A similar phenotype has been described in anergic T cells, which suggests that the effect of the *Vav1* mutations may in part mimic the anergic state (30). *Vav1*-deficient T cells produce more interferon-γ (IFN-γ) than do wild-type T cells in response to TCR stimulation (31). The abundance of the mRNA of *IFNγ* was higher in *Vav1*^{AA/AA} CD4⁺ T cells than in wild-type T cells and *Vav1*^{-/-} cells (Fig. 6C). We also examined transcription of the gene encoding tumor necrosis factor α (*TNFA*), another cytokine induced by stimulation of the TCR in T cells. In this case, the abundance of *TNFA* mRNA was lower in *Vav1*^{-/-} and *Vav1*^{AA/AA} T cells than in wild-type T cells, with the largest defect seen in *Vav1*^{AA/AA} cells. Finally, we examined induction of the expression of *CD25* because of the reduced abundance of CD25 protein on the surface of activated mutant T cells (Fig. 5, A and B). Once again we saw that the TCR-induced increase in the abundance of *CD25* mRNA was decreased in *Vav1*^{-/-} CD4⁺ T cells compared to that in wild-type cells

and was further reduced in *Vav1^{AA/AA}* cells (Fig. 6B). Thus, the GEF activity of Vav1 is necessary for TCR-dependent increases in the secretion of IL-2, and in the expression of *IFN γ* , *TNFA*, and *CD25*.

We were struck by the apparent similarity of these phenotypes to those seen in T cells deficient in the transcription factor NFAT1. *NFAT1^{-/-}* CD4⁺ T cells exhibit impaired induction of the expression of both *CD25* and *TNFA*, but normal induction of *Il2* expression in response to TCR stimulation (32-34). The similarity in phenotypes suggested that the GEF activity of Vav1 might be required to activate NFAT1. In agreement with this hypothesis, we found that both *Vav1^{-/-}* and *Vav1^{AA/AA}* CD4⁺ T cells were defective in induction of *NFAT2* mRNA, a known target of NFAT1 (Fig. 6B) (35).

The GEF activity of Vav1 is required for a subset of Vav1-dependent, TCR-induced signaling pathways

We have shown that the GEF activity of Vav1 is required for thymic selection and for TCR-induced activation of T cells. But what signaling pathways does this activity control? From studies of *Vav1^{-/-}* T cells, we and others have shown that Vav1 is required to transduce TCR signals that lead to a rise in the concentration of intracellular Ca²⁺ and to the activation of the kinases ERK, PKD1, and Akt (16-20). Measurement of the concentration of intracellular Ca²⁺ showed that TCR-induced Ca²⁺ flux was normal, or slightly elevated, in *Vav1^{AA/AA}* CD4⁺ and CD8⁺ T cells compared to that in wild-type cells, which was in contrast to the large defect seen in *Vav1^{-/-}* cells (Fig. 7A). Similarly, TCR-induced activation of ERK and PKD1 was normal in *Vav1^{AA/AA}* CD4⁺ T cells, but not in *Vav1^{-/-}* cells (Fig. 8A). Phosphorylation of LAT was not affected in either *Vav1^{AA/AA}* or *Vav1^{-/-}* T cells (Fig. 8A), in agreement with our previous study (36). *Vav1^{+/-}* T cells showed a mild decrease in TCR-induced Ca²⁺ flux compared to that in wild-type cells, but showed normal activation of ERK (Fig. 7B, 8B). These results show that Vav1 transduces TCR signals that lead to Ca²⁺ flux and to the activation of the ERK and PKD1 pathways independently of its exchange activity.

In contrast, TCR-induced activation of Rac1 was decreased in T cells from *Vav1^{AA/AA}* and *Vav1^{-/-}* mice compared to that in T cells from wild-type mice (Fig. 8C). This is consistent with Vav1 acting as a GEF for Rac1 in T cells, as it does in vitro. Furthermore, TCR-induced phosphorylation of Akt was strongly reduced in *Vav1^{AA/AA}* CD4⁺ T cells, to an extent similar to that seen in *Vav1^{-/-}* cells (Fig. 8A). This defect was not seen in *Vav1^{+/-}* T cells (Fig. 8B). The phosphorylation of Akt in T cells is completely dependent on the accumulation of the phosphatidylinositol-3,4,5-trisphosphate (PIP₃) (36), suggesting that the GEF activity of Vav1 is required for the generation of PIP₃. If the exchange activity of Vav1 regulates the production of PIP₃ through the activation of Rac GTPases, inhibition of these proteins should replicate the phenotype seen with the *Vav1^{AA}* mutation. We tested this hypothesis by stimulating CD4⁺ T cells through the TCR in the presence of either a Rac inhibitor (EHT1864) or a related inactive compound (EHT4063) (37). We found that inhibition of Rac GTPases strongly reduced TCR-induced phosphorylation of Akt, but not of ERK2, similar to the results obtained in *Vav1^{AA/AA}* T cells (Fig. 8D). Thus, we propose that a key function of the exchange activity of Vav1 is to activate Rac GTPases, with one of the downstream signaling pathways leading to the production of PIP₃.

The GEF activity of Vav1 is required for conjugate formation, integrin activation, and actin polymerization

Finally, we examined the role of the GEF activity of Vav1 in controlling formation of conjugates between T cells and antigen-presenting cells (APCs), cytoskeletal rearrangements, and T cell polarization, processes that are defective in the absence of Vav1

(17, 18, 21-23). In experiments with CD8⁺ T cells expressing a monoclonal F5 TCR, we measured the ability of the cells to form conjugates with APCs as a function of the dose of agonist peptide. We found that both *Vav*^{AA/AA} and *Vav*^{I^{-/-}} T cells had a similarly reduced ability to form conjugates, requiring about 10 times more peptide to achieve a conjugate efficiency equivalent to that of wild-type T cells, thereby demonstrating an important role for the GEF activity of Vav1 in this process (Fig. 9A). This defect in adhesion is likely to be due to a role for Vav1 in transducing inside-out signals that lead to the activation of the integrin lymphocyte function-associated antigen-1 (LFA-1) (21, 22). TCR-induced activation of LFA-1 was decreased in *Vav*^{AA/AA} T cells compared to that in wild-type T cells, although not as greatly as that in *Vav*^{I^{-/-}} cells (Fig. 9B). These results demonstrate that Vav1 transduces inside-out signals that lead to the activation of LFA-1 through both GEF-dependent and -independent functions.

Analysis of T cell:APC conjugates by confocal microscopy showed that the abundance of F-actin was reduced to a similar extent in *Vav*^{AA/AA} and *Vav*^{I^{-/-}} F5 CD8⁺ T cells, showing a critical role for the GEF activity of Vav1 in controlling actin polymerization (Fig. 9, C and D). In contrast, F-actin and LFA-1 polarization were not affected by either deletion of *Vav*1 or the introduction of the GEF mutant of Vav1 (Fig. 9, C and E). Lastly, we examined polarization of the microtubule organizing center (MTOC) in DP thymocytes that expressed the F5 TCR, a process that we have shown is dependent on Vav1 (21). We saw a clear defect in MTOC polarization in *Vav*^{I^{-/-}} cells, but not in cells expressing GEF-deficient Vav1 (Fig. 9, F, G, and H). Hence, we conclude that TCR-induced polarization of the MTOC in cell conjugates requires a GEF-independent function of Vav1, but not its GEF activity.

Discussion

To investigate the role of the GEF activity of Vav1 in T cell development and activation, we took great care to design a mutation that would eliminate exchange activity but leave the protein correctly folded and thus able to carry out potential non-GEF functions. Previous attempts to address the role of Vav1's GEF activity have used two types of mutations. In some cases, the DH domain has been entirely deleted (38, 39). Because this domain forms extensive interactions with the PH and C1 domains (3, 27), it is likely that deletion of the DH domain causes misfolding of the other domains and therefore could affect non-GEF functions of Vav1. Secondly, mutations have been made in conserved residues of DH domains. Many investigators have used a mutation in Leu²¹³, following a publication showing that mutation of this residue eliminates exchange activity (40). Several other studies have mutated residues in a conserved segment of the DH domain, amino acid residues 338 to 341 (18, 41). With the availability of a Vav1-Rac1 complex structure (3, 27), we can now see that Leu²¹³ and several of the residues in the 338 to 341 segment contribute to the structural integrity of the DH domain and not to direct interactions with the GTPase; thus, these mutations are likely to inactivate exchange activity by unfolding the DH domain. Again, this may perturb non-GEF functions of the protein. We believe that the mutation we describe here (Leu³³⁴→Ala, Lys³³⁵→Ala), is the first to eliminate the exchange activity of Vav1 without unfolding the DH domain or associated domains.

Another study has reported analysis of a mutation (Leu²⁷⁸→Gln) in the DH domain of Vav1, which caused loss of GEF activity *in vitro*, but had no effect on T cell development, T cell proliferation, cytokine production, and, notably, on TCR-induced activation of Rac1 (42). These results are difficult to reconcile with those that we report here, if the Leu²⁷⁸→Gln mutation has indeed eliminated GEF activity. Examination of the structure of Vav1 shows that Leu²⁷⁸ is located on the opposite side of the DH domain to its interface with Rac1, and the side chain of this residue does not appear to contribute directly to domain

stability (3, 27). Thus, it is hard to understand how mutation of this residue could affect GEF activity. Indeed, we note that this residue is not well conserved among DH domains and can sometimes be a glutamine, for example in the Dbl, Dbp, and Trio1 GEFs (43). Finally, mutation of the equivalent residue in the DH domain of Dbl (Gln⁵⁸⁶) had no effect on GEF activity (44). Taken together, these observations suggest that the Leu²⁷⁸→Gln mutation may not eliminate GEF activity, thereby explaining its lack of phenotype.

Our results show that although the *Vav1^{AA}* mutation affects both T cell development and activation, there are a number of Vav1-dependent processes for which the GEF activity of Vav1 is dispensable. These include the pre-TCR checkpoint during thymopoiesis, TCR-induced Ca^{2+} flux and activation of ERK and PKD1, and the polarization of the MTOC. Furthermore, our data show that although the GEF activity of Vav1 is required for efficient negative selection of DP thymocytes, there is also a role for non-GEF functions of Vav1 in this process. We have previously shown that Vav1 transduces TCR signals that lead to Ca^{2+} flux and the activation of ERK through the activation of phospholipase $\text{C}\gamma 1$ ($\text{PLC}\gamma 1$) and subsequent hydrolysis of phosphoinositide-4,5-bisphosphate (PIP_2) to generate the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (19, 20). Thus, our current results demonstrate that the TCR-induced activation of $\text{PLC}\gamma 1$ does not require the GEF activity of Vav1, but must involve a GEF-independent function. In earlier studies we showed that in the absence of Vav1, the TCR-induced association between $\text{PLC}\gamma 1$ and the adaptor protein SH2 domain-containing leukocyte phosphoprotein of 76 kD (SLP76) is greatly reduced (19). We propose that Vav1 may function as an adaptor protein, independent of its GEF activity, forming part of a complex of proteins that recruit $\text{PLC}\gamma 1$ to the plasma membrane. Interleukin-2 tyrosine kinase (Itk) also acts as an adaptor, bringing Vav1 to the plasma membrane, a role distinct from its function as an enzyme (45). Thus, it is likely that a signaling complex that brings $\text{PLC}\gamma 1$ to the membrane will include Vav1 and Itk and the adaptor proteins SLP76 and LAT. This GEF-independent regulation of $\text{PLC}\gamma 1$ by Vav1 may explain why overexpression of Vav1 in the Jurkat T cell line leads to an increase in the activation of NFAT even when the Vav1 bears a disabled DH domain (in which the sequence ³³⁸LLLQELV³⁴⁴ is mutated to ³³⁸IIIQDAA³⁴⁴) (41). The activation of NFAT is dependent in part on an increase in the concentration of intracellular Ca^{2+} , which leads to the activation of the phosphatase calcineurin, dephosphorylation of NFAT proteins, and their subsequent translocation to the nucleus (46). Thus, overexpression of either wild-type or DH-mutated Vav1 in Jurkat T cells may lead to activation of $\text{PLC}\gamma 1$, increased intracellular Ca^{2+} mobilization, and hence to enhanced NFAT activity in the nucleus.

The TCR-induced activation of PKD1 also required a non-GEF function of Vav1, because its activation was normal in *Vav1^{AA/AA}* T cells. Previous studies have shown that the antigen-receptor induced activation of PKD1 in B cells requires the activation of $\text{PLC}\gamma 2$ and the production of DAG (47). DAG in turn activates PKD1 in part through protein kinase C (PKC) enzymes. In T cells, TCR-induced activation of PKD1 also requires PKC activity (48) and, by analogy with B cells, may also require activation of $\text{PLC}\gamma 1$ and the production of DAG. Such a pathway would explain why Vav1, but not its GEF activity, is needed for the activation of PKD1.

We also showed that a GEF-independent function of Vav1 was required to transduce signals that led to MTOC polarization. Once again, this may be due to a role for Vav1 in controlling the activity of $\text{PLC}\gamma 1$, as this is critical for relocalization of MTOC (49). Although an earlier study suggested that $\text{PLC}\gamma 1$ -mediated Ca^{2+} flux might be needed for this polarization (50), another study showed that MTOC polarization in primary CD4^+ T cells is driven by the localization of DAG (49).

In view of the documented role for Rac1 and Rac2 at the pre-TCR checkpoint, we were surprised to find that the Vav1 GEF mutation did not lead to a strong block at this developmental stage. We and others have shown that a constitutively active Rac1 can promote differentiation of Rag1-deficient DN thymocytes that lack a pre-TCR to the DP thymocyte stage (51). In addition, elimination of both Rac1 and Rac2 leads to a strong block at the DN to DP transition stage (52). A possible explanation is that there are other GEFs for Rac1, such as Vav2 and Vav3 (53), that play a role at the pre-TCR checkpoint, and that these could provide enough Rac activation to enable differentiation to proceed normally in the *Vav1^{AA/AA}* mice.

Our studies have shown for the first time that the GEF activity of Vav1 is required for normal T cell development and activation. In particular, we have shown that this GEF activity is required to transduce TCR signals that lead to the phosphorylation of Akt. Furthermore, we showed that inhibition of Rac GTPases also led to a decrease in TCR-induced phosphorylation of Akt. Because this phosphorylation event is dependent on the accumulation of PIP₃, we propose that Vav1 transduces TCR signals through Rac1 to the generation of this key phosphoinositide. How Rac GTPases do this remains unclear, but it may be through direct activation of phosphoinositide-3-kinases (PI3Ks) (54, 55). The GEF activity of Vav1 is also required for the efficient formation of T cell:APC conjugates, most likely because of a contribution of this activity to the activation of integrins. However, we note that both GEF and non-GEF functions of Vav1 are required to transduce TCR signals that lead to the activation of integrins. The GEF-independent contribution may once again be through PLC- γ 1, because both Ca²⁺ flux and DAG-activated PKC enzymes have been implicated in the activation of integrins (56). In contrast, the GEF activity of Vav1 may promote integrin-mediated adhesion through Rho-family GTPases and reorganization of the actin cytoskeleton. Indeed, we showed that GEF activity was required for actin polymerization in T cells that formed conjugates with APCs.

We noted an apparent similarity in phenotype between *Vav1^{AA/AA}* and *NFAT1^{-/-}* CD4⁺ T cells, with both mutations causing reduced expression of *TNFA*, *CD25*, and *NFAT2*, but having no effect on the expression of *IL2*. A possible explanation for this may be that through Rac, PI3K, and Akt, the GEF activity of Vav1 leads to the phosphorylation and inhibition of glycogen synthase kinase 3B (GSK-3 β), a kinase that in turn phosphorylates NFAT1, thereby promoting its export from the nucleus and retention in the cytoplasm. Thus, loss of the GEF activity of Vav1 would result in a lower abundance of NFAT1 in the nucleus, despite there being normal TCR-induced flux of Ca²⁺.

In a number of assays we noted that *Vav1^{AA/AA}* T cells were more affected than were *Vav1^{-/-}* T cells. These included TCR-induced proliferation and induction of the expression of *IFN γ* , *TNFA*, and *CD25*. This difference may be due to compensation in *Vav1^{-/-}* T cells by the other family members Vav2 and Vav3, which may not occur as readily in cells expressing a GEF-inactive Vav1, because the latter may block access to TCR signaling pathways of the other Vav-family proteins.

The results presented here give us the opportunity to re-interpret our findings in *Vav1^{R422G/R422G}* mice, which carry a point mutation in the PH domain of Vav1 (36). This mutation results in reduced TCR-induced Ca²⁺ flux and activation of ERK and Akt. Because our current data show that only the latter process is GEF-dependent, we deduce that the PH mutation has affected both the GEF activity of Vav1 and a GEF-independent function. The PH domain of Vav1 is proposed to bind to PIP₂ and PIP₃, and the R422G mutation eliminates this binding (57). Together, these results suggest that binding of phosphoinositides to the PH domain of Vav1 may be required for two independent functions, first, for full activation of exchange activity, and, second, for a putative adaptor

role for Vav1. In both cases, the binding of phosphoinositides to the PH domain may contribute to these functions by mediating the recruitment of Vav1 to the membrane or by affecting its orientation at the membrane after its recruitment there by other domains.

In conclusion, we have shown for the first time that the exchange activity of Vav1 is important for T cell development and activation, and is required to transduce TCR signals to the production of PIP₃, the activation of Rac1, Akt, and integrins, and to the polymerization of actin. Furthermore, our results also indicate unequivocally that Vav1 has GEF-independent functions, at least one of which is to transduce signals to the activation of PLC γ 1. More generally, our study suggests that drugs targeting the GEF activity of Vav1 may be therapeutically useful to dampen Vav1-dependent responses, and demonstrates a genetic approach that can be used to predict therapeutic and side effects of drugs that target other GEFs.

Materials and Methods

Mice

Mice carrying the *Vav1*^{AA} allele were generated essentially as described previously (36). An almost identical targeting vector was used, except that it carried a mutation in exon 10 to introduce the Leu³³⁴→Ala, Lys³³⁵→Ala amino acid mutations. The mutations were introduced with the oligonucleotides 5'-GATGGTACCTATGCAACGCGTGGCGGCCTATCACCTCCTTCTCCAG-3' and 5'-TCATGTTGACACAGTCGAAAATCAT-3', by standard methods. Along with the designed amino acid substitutions, the mutant allele contains silent mutations that introduce an *Afl*III site to assist screening and to disrupt a stable hairpin structure in the oligonucleotide used for mutagenesis. PC3 embryonic stem (ES) cells (129S4 genetic background) were electroporated with the targeting vector (58), and resulting G418-resistant clones were screened by Southern blotting analysis of their DNA with two probes lying outside the homology arms of the targeting construct and one probe specific for the *neo* gene. At least two digests were used for each probe to confirm structural integrity and the presence of the designed mutation in the *Vav1* locus. Correctly targeted ES clones were injected into C57BL/6 blastocysts and the resultant chimeric male mice from two independent ES lines (3H5 and 6H12) were bred to 129S8 females. Cre recombinase activity in the male germline resulted in deletion of the loxP-flanked *neo* gene and generation of mice carrying the *Vav1*^{AA} (*Vav1*^{tm4Tyb}) allele. The presence of the mutation in mice was confirmed by polymerase chain reaction (PCR) assay with the oligonucleotides 5'-CATCAGGGGTCAACCTGC-3' and 5'-CTTCCCAGGCACGCACTT-3' followed by sequencing of the PCR product or its digestion with *Afl*III or *Tau*I restriction endonucleases. *Vav1*^{AA/+} mice from line 3H5 were backcrossed with 129S8 mice for 2 generations or with B10.BR mice for 4 generations and then intercrossed to generate homozygous *Vav1*^{AA/AA} mice. *Vav1*^{AA/+} mice from ES line 6H12 were backcrossed with 129S8 for 10 generations and intercrossed to generate homozygous *Vav1*^{AA/AA} mice. The experiments were carried out interchangeably with mice derived from ES lines 3H5 and 6H12. Both lines produced comparable results. For all experiments on mice with the B10.BR background, wild-type mice were littermates. The generation of mice carrying a mutation disrupting the *Vav1* gene (*Vav1*^{tm1Tyb/tm1Tyb}; *Vav1*^{-/-}) on both a 129S8 and B10.BR background has been previously described (15). Mice bearing the F5 TCR transgene (28), mice bearing the TCR7 transgene (29), and mice deficient for Rag1 (*Rag1*^{-/-}) (59) were obtained from D. Kioussis, A. O'Garra, and D. Baltimore, respectively. All mice were analyzed between six and eight weeks of age except when biochemical analysis was performed, in which case the mice were between six and nine weeks of age.

Antibodies and flow cytometry

Biotin and fluorochrome-conjugated antibodies against CD3 ϵ , CD4, CD5 CD8 α , CD11c, CD25, CD44, B220, Mac1, Dx5, Gr1, Ter119, TCR β , V β 5, V β 8, V β 11, V β 12, TCR $\gamma\delta$, Thy1.2, and Streptavidin-PerCP were purchased from BD Pharmingen and eBioscience. Antibody against α -tubulin was purchased from Sigma Aldrich, antibody against integrin α L (clone I21/7) was from Santa Cruz, and Alexa Fluor 488-conjugated antibody against mouse immunoglobulin G1 (IgG₁) and Alexa Fluor 647-conjugated antibody against rat IgG were from Invitrogen. Single-cell suspensions from various organs were incubated in phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), 0.01% NaN₃ containing the appropriate antibodies. For analysis of DN subsets, thymocytes were incubated with antibodies against lineage (Lin) markers (CD4, CD8, CD3 ϵ , TCR β , TCR $\gamma\delta$, B220, Mac1, Gr1, and Dx5), Thy1.2, CD44, and CD25 to enable identification of DN1 (Lin⁻Thy1.2⁺CD44⁺CD25⁻), DN2 and DN3 (Lin⁻Thy1.2⁺CD25⁺), and DN4 (Lin⁻Thy1.2⁺CD44⁻CD25⁻) subsets. Cell detection was performed on a FACSCalibur (BD Biosciences) flow cytometer, after which analysis was performed with FlowJo software (Tree Star, Inc.).

Proliferation assays

Naïve CD4⁺CD44^{lo} T cells from peripheral and mesenteric lymph nodes were purified by negative selection. Single-cell suspensions were incubated with biotin-conjugated antibodies against CD8, CD11c, CD25, CD44, B220, Mac, DX5, Gr1, and Ter119 and were then washed and incubated with Streptavidin-conjugated magnetic beads (Dynabeads, Invitrogen). Purity of CD4⁺CD44^{lo} T cells (typically >90%) was confirmed by flow cytometry. Cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (1.5 μ M), resuspended in proliferation medium [RPMI 1640 medium, 10% fetal calf serum (FCS), 50 μ M 2-mercaptoethanol], and cultured with various amounts of plate-bound antibody against CD3 ϵ (145.2C11, BD Biosciences) in the presence or absence of antibody against CD28 (1 μ g/ml, 37.51, eBioscience) for 72 hours. After culture, cells were incubated with antibodies against CD4 and CD25 and with 7-AAD to exclude dead cells and were analyzed by flow cytometry. The percentage of cells at the start of the culture that had divided at least once by the end of the culture period was calculated with FlowJo software.

Detection of IL-2 and reverse transcriptase (RT)-PCR

CD4⁺CD44^{lo} T cells from peripheral and mesenteric lymph nodes were purified by negative selection as described earlier. After culturing the cells for 48 hours in proliferation medium in the absence or presence of various amounts of plate-bound antibody against CD3 ϵ and 1 μ g/ml of antibody against CD28, the amount of IL-2 in supernatants from four replicas for each condition was determined by enzyme-linked immunosorbent assay (ELISA) with a Mouse IL2 ELISA Ready-SET-Go kit (eBioscience). To analyze the amount of mRNA in cells, CD4⁺CD44^{lo} T cells were cultured for 6 or 24 hours in proliferation medium alone or in the presence of various amounts of plate-bound antibody against CD3 ϵ and 1 μ g/ml of antibody against CD28. RNA was prepared with the RNeasy Mini kit (QIAGEN), treated with DNase (Ambion), converted into cDNA by standard protocols, and quantified by TaqMan Gene Expression Assays (Applied Biosystems). In each sample, the expression of the genes being analyzed was normalized to that of the gene encoding hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1).

Measurement of Ca²⁺ flux

Ca²⁺ flux in lymph node cells labeled with PerCP-conjugated antibody against CD4, fluorescein isothiocyanate (FITC)-conjugated antibody against CD8, phycoerythrin (PE)-conjugated antibody against CD44, antibody against CD3 ϵ (10 μ g/ml), and Indo-1-AM (2

μg/ml, Invitrogen) was measured with LSR and LSRII flow cytometers (BD Biosciences) as previously described (19).

Stimulation of CD4⁺ T Cells for biochemical analysis

Naïve CD4⁺CD44^{lo} T cells from peripheral and mesenteric lymph nodes were purified by negative selection as described earlier. Stimulation of the cells through the TCR by crosslinking with antibody against CD3e (10 μg/ml) and antibody against CD28 (10 μg/ml) and subsequent Western blotting analysis were performed essentially as described previously (19). To study the effect of inhibiting Rac1, the CD4⁺CD44^{lo} T cells were pre-incubated for 7 min at 37°C with either the Rac1 inhibitor EHT1864 or with the control compound EHT4063 (gifts of L. Desire, ExonHit Therapeutics) prior to stimulation. Vav1 was immunoprecipitated with an antibody against Vav1 (C-14, Santa Cruz) by standard techniques. The following antibodies were used for detection of proteins by Western blotting: anti-phosphotyrosine (4G10, Upstate Biotechnology), anti-phospho-ERK (E-4), anti-Vav1 (C-14), anti-Akt (B-1) from Santa Cruz Biotechnology, anti-phosphoserine473-Akt, and anti-phosphotyrosine171-human LAT (equivalent to pY175 murine LAT) from Cell Signaling Technology, anti-ERK2 (gift from C. Marshall), anti-LAT (gift from M. Turner), and anti-PKD1 and anti-phosphoserine916-PKD1 (both gifts from D. Cantrell). Antibody binding was revealed with IRDye800-conjugated goat antibody against mouse IgG (Rockland) for mouse antibodies, or Alexa Fluor 680-conjugated goat antibody against rabbit IgG (Invitrogen) for rabbit polyclonal antibodies. To control for equal loading across lanes, blots were either directly incubated with antibody against the appropriate total protein (for Akt, Vav1, and ERK) or, for antibodies against phospho-LAT and phospho-PKD1, blots were stripped and reincubated with antibodies against LAT and PKD1, respectively. Signals were detected with an Odyssey Infrared Imager (Li-Cor Biotechnology) and analyzed with the manufacturer's software.

Activation of Rac1 in T cells

CD4⁺ T cells from peripheral and mesenteric lymph nodes were purified by negative selection as described earlier. The cells were cultured in proliferation medium at 4×10^6 cells/ml with 5 μg of plate-bound antibody against CD3e and 2.5 μg of antibody against CD28 for 48 hours. Live cells were purified by centrifugation through a Lympholyte-M (Cedarlane Laboratories) gradient and expanded in cultures containing 15 ng/ml of murine IL-2 (Peprotech) for 48 hours. Cells were rested in proliferation medium without IL-2 for 6 hours and stimulated by crosslinking with antibodies against CD3e and CD28 as described previously. 8 to 10×10^6 cells were lysed for 10 min in 110 μl of 25 mM tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% NP-40, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) containing 2 nmol of bacterially expressed glutathione *S*-transferase (GST)-Pak1-Rac-binding domain (RBD), a fusion protein of GST and amino acid residues 1 to 252 of rat Pak1. Cell lysates were cleared by centrifugation at 15,340g at 4°C for 10 min and 40 μl of 50% glutathione Sepharose slurry (GE Healthcare) was added. Samples were incubated at 4°C for 40 min, the Sepharose was washed four times in 1% Triton X-100 (Sigma), 25 mM tris-Cl (pH 7.5), 320 mM NaCl, 10 mM MgCl₂, 1 mM PMSF, and GST-Pak1-RBD fusion protein, and any associated Rac1-GTP was eluted with Laemmli sample buffer for 5 min at 95°C. Samples were analyzed by Western blotting as described earlier. Western blots were incubated with an antibody against Rac1 (23A8, Millipore) and reactive proteins were identified as described earlier. Three independent experiments were analyzed. Within each experiment, the ratios of Rac1-GTP to Rac1 in the 12 samples (four time points for each of three genotypes) were normalized to the mean of these 12 ratios, which was set at 1. Finally, the 3 experiments were combined to generate mean ± standard error of the mean (SEM) ratios for each of the 12 data points.

Exchange activity and thermal stability of DH domains

The wild-type and Leu³³⁴→Ala, Lys³³⁵→Ala (AA) mutant DH domains (amino acid residues 190 to 400) were expressed and purified as described previously (3). The rate of nucleotide exchange was measured by fluorescence as previously described (3) with 30 μ M of either the wild-type or mutant DH domain and 300 nM Rac1-(2',3')-O-(*N*-methylantraniloyl)-GDP (mant-GDP). Circular dichroism (CD) measurements were performed with a Jasco J-715 spectropolarimeter fitted with a PTC 348-WI Peltier unit. Measurements were carried out in a buffer containing 50 mM Hepes (pH 7.5) with a protein concentration of 10 μ M in cuvettes with a 1-mm path length (Hellma). The CD spectrum was measured at 25°C and thermal unfolding curves were obtained by measuring molar ellipticity at 220 nm while heating at a rate of 1°C/min.

Formation of T cell:APC conjugates

F5 CD8⁺ T cells from peripheral and mesenteric lymph nodes of *Rag1*^{-/-} mice carrying the F5 TCR transgene and the appropriate *Vav1* allele were purified by negative selection as described earlier. B cells from polyclonal wild-type mice were purified similarly with biotin-conjugated antibodies against TCR β and CD43. B cells were incubated with different doses of NP68 peptide for 30 min at 37°C. T and B cells were mixed at a 1:1 ratio, and conjugates were allowed to form for 1 hour at 37°C. Nonspecific interactions were disrupted by pipetting the cell suspensions up and down twelve times, conjugates were fixed with 3% paraformaldehyde (Thermoscientific), incubated with fluorochrome-conjugated antibodies against CD8 and B220, and analyzed by flow cytometry. Each data point was calculated as a mean of triplicate samples.

Cell adhesion

Naïve CD4⁺CD44^{lo} T cells from peripheral and mesenteric lymph nodes were purified by negative selection as described earlier. Adhesion assays were carried out with a published protocol (60). The cells were labelled with CFSE (1 μ M) and resuspended in Hank's Balanced Salt Solution (Invitrogen), 1% BSA at a concentration 1.4×10^6 cells/ml. 50 μ l of cell suspension were mixed with 50 μ l of antibody against CD3 ϵ (0.2 μ g/ml) and incubated for 5 min at 37°C in V-bottom plates precoated with intercellular adhesion molecule 1 (ICAM-1, 3 μ g/ml in PBS, R&D Systems). Plates were centrifuged at 200g for 1 min and CFSE fluorescence was read at the bottom of the wells by the Tecan Safire II fluorescence reader (MTX Lab Systems, Inc). Adhesion was normalized by subtracting adhesion seen in the absence of stimulation and by setting adhesion induced by 2 mM MnCl₂ at 100%.

Imaging of conjugates

F5 CD8⁺ T cells from lymph nodes and spleens were cultured at 1×10^6 cells/ml in the presence of 100 nM NP68 peptide for 72 hours. Dead cells were removed with Lympholyte-M (Cedarlane Laboratories). Viable lymphocytes were resuspended at 0.2×10^6 cells/ml and cultured for a further 96 hours in the presence of 15 ng/ml of IL-2 (Peprotech) and then rested for 6 hours in the absence of IL-2. Purified splenic B cells were stained with 2.5 μ M 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) (Invitrogen) according to the manufacturer's protocol. Rested T cells or naïve DP thymocytes from F5 TCR transgenic mice (0.4×10^6 in each case) were seeded onto coverslips coated with poly-L-lysine (Sigma) and allowed to adhere for 15 min at room temperature. CMTMR-stained B cells (0.8×10^6), loaded with 1 μ M NP68 peptide were added, centrifuged at 260g for 3 min at room temperature, and incubated for the remaining time period at 37°C. Cells were fixed with 3% paraformaldehyde for 15 min at room temperature, washed with PBS, permeabilized with 0.2% Triton X-100 for 2 min, blocked with PBS containing 5% FCS and 3% BSA for 15 min, and incubated with primary antibodies against the indicated antigens or

with Alexa Fluor 647–phalloidin (Invitrogen) for 1 hour at room temperature. Cells were washed, incubated with secondary antibodies for 45 min, washed, stained with Hoechst 33342 (5 µg/ml) for 2 min, washed, and mounted in Mowiol 4-88 reagent (Calbiochem). Confocal microscopy was performed with a Leica TCS SP2 microscope with a 63x objective (NA 1.32). Images were analyzed with Cell Profiler software or a custom-made plugin to LabView software developed by Dr Yan Gu, NIMR. Polarization of F-actin or LFA-1 was calculated as a ratio of mean fluorescence intensity (MFI) at the APC contact site versus the MFI of the rest of the cell. Polarization of the MTOC in thymocytes was scored by determining whether the MTOC was located in the third of the cell nearest to the APC (proximal), that furthest from the APC (distal), or in the middle third of the cell.

Statistical analysis

All statistical comparisons were carried out with the nonparametric two-tailed Mann-Whitney test except that the Student's *t* test was applied to analysis of the production of IFN- γ and Fisher's exact test was applied to analysis of MTOC polarization.

Acknowledgments

We thank D. Kioussis, A. O'Garra, and D. Baltimore for mouse strains; C. Marshall, M. Turner, and D. Cantrell for antibodies; L. Desire (ExonHit Therapeutics) for EHT1864 and EHT 4063; R. Köchl for help with image analysis; J. Janzen and S. Sriskantharajah for technical advice; Biological Services at NIMR for animal husbandry; and S. Ley for critical reading of the manuscript. AS was funded by a grant from the Leukaemia Research Fund. LV, OK, JR, SS, KR, and VT were funded by the Medical Research Council programme number U117527252.

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Fig. 1.

Generation of mice expressing GEF-inactive Vav1. (A) Vav1 GEF mutants were originally designed on the basis of the structure of the complex between the DH and PH domains of Tiam1 and Rac1 (PDB ID:1FOE) (25) and of the DH domain of Vav1 (PDB ID:1F5X) (26), and confirmed on the basis of the structure of the complex between the DH-PH-C1 region of Vav1 and Rac1 (PDB ID:2VRW) (3). The left panel shows a structural overlap of these complexes based on least-squares superposition of the respective DH domains with the PH and C1 domains of Vav1 and the PH domain of Tiam1 removed for clarity. The DH domain of Vav1 is shown in cyan and Rac1 is colored blue. The Switch II region of Rac1 is highlighted in red and the DH and Rac1 components of the Tiam1 structure are shown in gray. The right panel shows a magnified view of the DH-Rac1 interactions around switch II. The sidechains of Leu³³⁴ and Lys³³⁵ of Vav1 and of Leu¹¹⁹⁴ and Lys¹¹⁹⁵ of Tiam1 are shown in stick representation. In both complexes, these residues make crucial nonpolar and hydrogen-bonding interactions with Switch II of the small GTPase, but appear to make no structurally important interactions with the remainder of the DH domain. (B) The graph on the left shows the circular dichroism spectra of wild-type (WT) or mutated (AA) recombinant DH domains of Vav1 at 25°C. The two curves are so similar that it is hard to see them apart, indicating that the WT and mutant domains have indistinguishable secondary structure. The graph on the right shows the molar circular dichroism at 220nm of the DH domains as a function of temperature. The change in circular dichroism as the temperature is increased is a measure of the thermal stability of the domain. (C) The time-dependent change in normalized fluorescence of mant-GDP, which had been pre-loaded onto recombinant Rac1. Release of mant-GDP results in a decrease in fluorescence. Rac1 was either incubated alone or in the presence of the WT or the AA mutant DH domain of Vav1. (D) Diagram showing part of the WT *Vav1* genomic locus before gene targeting (*Vav1*^{WT}),

the *Vav1* gene after insertion of loxP-flanked *neo* gene into intron 9 and of the Leu³³⁴→Ala,Lys³³⁵→Ala (LK334AA) mutation into exon 10 (*Vav1*^{AAneo}), and of the final targeted *Vav1* allele after Cre-mediated removal of the *neo* gene, leaving behind a single loxP site in intron 9 and the LK334AA mutation in exon 10 (*Vav1*^{AA}). Black boxes indicate *Vav1* exons, black triangles indicate loxP sites, and arrows indicate the positions of oligonucleotides used to identify the mutant allele by PCR. Dotted lines indicate the extent of homology used in the targeting vector. Restriction sites: R, *EcoRI*; S, *SpeI*; X, *XhoI*. (E) Electrophoretogram showing the PCR products that were used to identify *Vav1*^{WT} (WT, 496 bp) and *Vav1*^{AA} (AA, 571 bp) alleles, from tail DNA of mice of the indicated genotypes. The sizes of marker (M) bands are indicated. (F) Predicted DNA and protein sequence around the introduced AA mutation. Altered nucleotides and amino acids are indicated in red. In addition to the nucleotide changes required to mutate amino acids Leu³³⁴ and Lys³³⁵, three further silent nucleotide changes were made in order to introduce an *Afl*III site and to disrupt a stable hairpin in the oligonucleotide used for mutagenesis. The graph below shows the fluorescence trace and deduced sequence from around the site of mutation in DNA amplified by PCR from the tail of a *Vav1*^{AA/AA} mouse. Nucleotides mutated from the WT are underlined in red.

Fig. 2.

Quantitation and phosphorylation of Vav1 in *Vav1^{AA/AA}* T cells. **(A)** Western blots of total cytoplasmic extracts of naïve CD4⁺CD44^{lo} lymph node T cells from mice of the indicated genotypes incubated with antibodies against Vav1, α-tubulin, LAT, and ERK2. The graph shows the mean ± SEM amount of Vav1 in naïve CD4⁺ lymph node T cells from mice of the indicated genotypes relative to those of α-tubulin, ERK2, and LAT normalized to that in wild-type (WT) T cells, which was set to 1. **(B)** Western blot analysis of immunoprecipitated Vav1 from naïve CD4⁺ lymph node T cells from mice of the indicated genotypes stimulated through CD3e and CD28 for the indicated times. Western blots were incubated with antibodies against phosphotyrosine (to detect pVav1) and Vav1. The graph shows the relative abundance of pVav1, determined from a ratio of the intensity of pVav1 to that of total Vav1.

Fig. 3.

The exchange activity of Vav1 is required for normal T cell development. (A) Contour plots showing separation of CD4⁺CD8⁻ double negative (DN) thymocytes into DN1 (CD25⁻CD44⁺), DN2/3 (CD25⁺CD44⁺), and DN4 (CD25⁺CD44⁻) subsets in mice of the indicated *Vav1* genotypes. Numbers on these and subsequent plots indicate the percentages of cells that fell into the gates. Graphs show mean ± SEM numbers of total thymocytes and of cells in the DN subsets. Numbers here and in subsequent figures indicate millions of cells. (B) Contour plots showing CD4 and CD8 on thymocytes from mice of the indicated genotypes. Gates identify double negative (DN, CD4⁻CD8⁻), double positive (DP, CD4⁺CD8⁺), and CD4⁺ or CD8⁺ single positive (CD4SP, CD4⁺CD8⁻ and CD8SP, CD4⁻CD8⁺) thymocytes. For quantification of CD4SP and CD8SP cells only TCRβ^{hi} cells were taken into account. Graph shows mean ± SEM numbers of cells in the indicated subsets. (C) Histograms showing CD5 on DP thymocytes. (D) Contour plots of CD4 and CD8 in the lymph nodes of mice of indicated genotypes. Gates identify CD4⁺ and CD8⁺ T cells. Graph shows mean ± SEM numbers of CD4⁺ and CD8⁺ T cells in the spleen and lymph nodes (LN). (E) Contour plots showing CD44 and CD25 on CD4⁺ T cells from the lymph nodes of mice of the indicated genotypes. Gates identify populations of naïve (CD25⁻CD44^{lo}), activated or memory (CD25⁺CD44^{hi}), and regulatory (CD25⁺CD44⁺) CD4⁺ T cells. Graph shows mean ± SEM numbers of the corresponding T cell populations. (F) Histograms showing CD44 on CD8⁺ T cells from the lymph nodes of mice of the indicated genotypes. Gates identify populations of naïve (CD44^{lo}) and activated or memory

(CD44^{hi}) CD8⁺ T cells. Graph shows mean $\pm$ SEM numbers of the corresponding T cell populations. **(G)** Histograms showing TCR β on naïve T cells from the lymph nodes of mice of the indicated genotypes. Colors used are as described for Panel C. **(H)** Graphs showing mean $\pm$ SEM numbers of CD4SP and CD8SP thymocytes, and CD4⁺ and CD8⁺ T cells in the lymph nodes of WT and *Vav1*^{+/-} mice. N = 6 for all parts of this figure. *, $P < 0.05$; **, $P < 0.01$.

Fig. 4.

The exchange activity of Vav1 is required for positive and negative selection of thymocytes. (A) Contour plots of CD4 and CD8 on thymocytes from mice carrying the F5 TCR transgene that were also *Rag1^{-/-}* and had the indicated *Vav1* genotype. Numbers above the plots show mean ± SEM thymic cellularity (x 10⁶). Graphs show mean ± SEM numbers of CD8SP thymocytes and CD8⁺ lymph node (LN) T cells, and the ratio of CD8SP:DP thymocytes. (B) Contour plots of CD4 and CD8 on thymocytes from mice carrying the TCR7 TCR transgene. Graphs show CD4SP and CD8SP thymocyte numbers and the ratio of CD4SP:DP cells. (C) Contour plots showing CD4 and CD8 in the lymph nodes of mice described in panel B. Gates identify CD4⁺ and CD8⁺ T cells. Graphs show mean ± SEM numbers of these T cells in the spleen and lymph nodes. (D) Graph shows the mean ± SEM percentages of CD4SP thymocytes expressing a TCR with the indicated Vβ chain in mice of the indicated genotypes. N = 8 for all parts of this figure. *, *P* < 0.05; **, *P* < 0.01.

Fig. 5.

The absence of the exchange activity of Vav1 results in severely compromised activation and proliferation of T cells. Naïve CD4⁺CD44^{lo} lymph node T cells from mice of the indicated genotypes were prelabeled with CFSE and stimulated for 72 hours with the indicated doses of plate bound antibody against CD3 ϵ in the presence or absence of soluble antibody against CD28 (1 μ g/ml). Histograms show forward scatter (FSC) as a measure of cell size, staining of CD4 and CD25, and CFSE fluorescence. Graphs show the mean percentage of cells in triplicate samples that had high FSC, high abundance of CD4, or were positive for CD25. Percentage of cells in the initial population that had divided at least once by the end of culture period was determined by analysis of CFSE dilution as described in the Materials and Methods. Comparison of WT and Vav1^{AA/AA} T cells with (A, B) Vav1^{-/-} or (C) Vav1^{+/+} T cells. Note that experiments shown in (A) and (B) were carried out with plates with different binding capacities for antibodies and are therefore not directly comparable.

Fig. 6.

Expression of selected genes is severely perturbed in T cells with compromised Vav1 exchange activity. (A) Graphs show mean $\pm$ SEM amounts of IL-2 protein in culture supernatants of CD4⁺ T cells that were either unstimulated (-) or were activated with the indicated doses of plate-bound antibody against CD3 ϵ (n = 4). (B) Mean $\pm$ SEM amounts of mRNA for *Il2*, *Tnf α* , *Il2ra* (CD25), and *NFAT2* in CD4⁺ T cells of the indicated genotypes that were either unstimulated (-) or were activated (+) for 6 hours with plate-bound antibody against CD3 ϵ (220 ng/well) (n = 5). (C) Mean $\pm$ SEM abundance of *IFN γ* mRNA in CD4⁺ T cells of the indicated genotypes that were activated for the indicated times with plate-bound antibody against CD3 ϵ (1 μ g/well) (n = 3). In all experiments, stimulation with antibody against CD3 ϵ was carried out in the presence of soluble antibody against CD28 (1 μ g/ml). *, $P < 0.05$; **, $P < 0.01$.

Fig. 7. TCR-induced Ca^{2+} flux is independent of Vav1 GEF activity. Graphs show ratio of Indo1 violet/blue fluorescence as a measure of the concentration of intracellular Ca^{2+} as a function of time in naïve ($CD44^{lo}$) $CD4^{+}$ or $CD8^{+}$ lymph node T cells from mice of the indicated genotypes. Arrows indicate the times at which cells were stimulated with antibody against CD3 ϵ . (A) Comparison of WT and $Vav1^{-/-}$ T cells with $Vav1^{AA/AA}$ cells or (B) $Vav1^{+/-}$ T cells.

Fig. 8.

The GEF activity of Vav1 is required for a subset of TCR-induced signaling pathways. (A, B) Western blots of total cytoplasmic extracts of naïve CD4⁺CD44^{lo} lymph node T cells from mice of the indicated genotypes that were stimulated through CD3e and CD28 for the indicated times. Western blots were incubated with antibodies against phosphorylated forms of ERK2, PKD1, Akt, and LAT, and with antibodies against total ERK2, PKD1, Akt, and LAT. The graphs on the right show the relative abundance of phosphorylated forms of ERK2, PKD1, Akt, and LAT, as determined from a ratio of the intensity of the bands of phosphorylated proteins to those of total proteins. Comparison of *Vav1*^{MAA/MAA} and *Vav1*^{-/-} T cells with (A) WT or (B) *Vav1*^{+/+} T cells. (C) Western blots of active Rac1-GTP pulled down with a GST-Pak1-RBD fusion protein and of total cytoplasmic lysates from CD4⁺ T cells stimulated through CD3e and CD28 for the indicated times. Western blots were incubated with an antibody against Rac1. Graph shows mean ± SEM ratios of Rac1-GTP to total Rac1 of three independent experiments, normalized as described in Materials and Methods. (D) Western blots and graphs (presented as described for panel A) of WT naïve CD4⁺ lymph node T cells treated with either 20 μM or 40 μM of the Rac inhibitor EHT 1864 or the inactive control compound EHT 4063.

Fig. 9.

Formation of APC:T cell conjugates and polymerization of actin require the GEF activity of Vav1. **(A)** The graph in the left panel shows the mean $\pm$ SEM percentages of F5 CD8⁺ T cells found in conjugates with APCs as a function of the concentration of the NP68 peptide. The graph in the right panel shows the mean $\pm$ SEM percentages of F5 CD8⁺ T cells in conjugates with APC cells loaded with a nonspecific peptide (gag) at the highest dose tested for NP68 (5 nM). **(B)** Graph shows the mean $\pm$ SEM percentages of CD4⁺ T cells of the indicated genotypes that had adhered to plates coated with ICAM-1 after stimulation through CD3e ($n = 6$). Adhesion was normalized by setting the adhesion of cells seen in the absence of antibody against CD3e to 0% and that in response to Mn^{2+} to 100%. **(C)** Confocal single optical sections of pre-activated and rested F5 CD8⁺ T cells conjugated for 7 min with NP68 peptide-pulsed and CMTMR-stained APCs. Cells were stained for F-actin with phalloidin; nuclei were visualized with Hoechst. Scale bar, 5 μm . **(D)** Graph of the mean $\pm$ SEM amounts of F-actin (arbitrary units) in F5 CD8⁺ T cells quantified from images such as those shown in panel C (number of conjugates analyzed: $n = 32$). **(E)** Mean $\pm$ SEM polarization of F-actin and LFA-1 within F5 CD8⁺ T cells towards APCs quantified as described in the Materials and Methods (number of cells analyzed: $n = 27$). **(F)** Confocal single optical sections of DP thymocytes expressing the F5 TCR, conjugated for 20 min with NP68 peptide-pulsed and CMTMR-stained APCs. Cells were immunostained for α -tubulin and CD4 (to identify DP cells). Nuclei were stained with Hoechst. Arrows indicate position of the MTOC. Scale bar, 5 μm . **(G)** Schematic representation of the analysis of MTOC movement. **(H)** Graph shows distribution of the MTOC within DP thymocytes conjugated as described in panel F and classified as described in panel G according to whether the MTOC was located proximal or distal to the APC, or was in the middle of the thymocyte. Statistical differences in MTOC distribution between the proximal and middle and distal positions were determined by Fisher's exact test (number of conjugates analyzed: $n = 34$). *, $P < 0.05$; **, $P < 0.01$.