Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Strongly reduced alloreactivity and long-term survival times of cardiac allografts in Vav1- and Vav1/Vav2-knockout mice

Gisbert Weckbecker,[1] Christian Bruns,[1] Klaus-Dieter Fischer,[2] Christoph Heusser,[1] Jianping Li,[1] Barbara Metzler,[1] Randall E. Morris,[1] Barbara Nuesslein-Hildesheim,[1] Friedrich Raulf,[1] Grazyna Wieczorek[1] and Gerhard Zenke[1]

1 Autoimmunity and Transplantation, Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland
2 Universität Ulm, Ulm, Germany

Keywords:
Vav, alloreactivity, graft, T-cell, antibody, mouse

Correspondence
Prof. Gisbert Weckbecker, PhD, Autoimmunity and Transplantation, Novartis Institutes for BioMedical Research, WSJ-386.628, CH-4002 Basel, Switzerland. Tel.: +41 61 3247936; fax: +41 61 3243576; e-mail: gisbert.weckbecker@novartis.com

Received: 28 August 2006
Revision requested: 29 September 2006
Accepted: 14 November 2006

doi:10.1111/j.1432-2277.2006.00438.x

Summary

Vav proteins mediate T- and B-cell activation by functioning as GTP/GDP exchange factors for small GTPases. We have studied the role of Vav1 and Vav2 in allogeneic T-cell activation, antibody responses and allograft rejection. Alloantigen-induced proliferation of T cells from Vav1- and Vav1/Vav2-knockout (ko) mice was decreased by >90% in a mixed lymphocyte reaction. In whole-blood cultures, Vav deficiency led to markedly impaired T- and B-cell activation. Expansion of Vav1- or Vav1/Vav2-ko T cells (C57BL/6) was reduced after transfer into severe combined immune deficiency/beige recipient mice (BALB/c). After priming with 2,4-dinitrophenyl (DNP)–keyhole limpet hemocyanin, T-cell-dependent anti-DNP IgM and IgG antibody levels were normal in Vav1-ko mice but undetectable in Vav1/Vav2-ko mice. The median survival time of BALB/c cardiac allografts transplanted into C57BL/6 Vav1-ko mice (n =13) or Vav1/Vav2-ko mice (n = 5) was >100 and >77 days, compared with 8–9 days in the corresponding wild-type mice. Vav1/Vav2-ko mice with <100 days graft survival developed bacterial skin infections and were prematurely killed with beating cardiac allograft. Long-term surviving transplants of single and double ko mice showed mild cellular interstitial rejection and mild to severe vascular remodeling. In conclusion, our studies show for the first time that the absence of Vav1 and Vav1/Vav2 in ko mice strongly reduces alloreactivity and results in long-term allograft survival, whereas antibody responses were only affected in Vav1/Vav2 ko mice.

Introduction

Three structurally related Vav proteins, Vav1–3, have been identified in mammals [1–3]. While Vav1 is expressed primarily in cells of the hematopoietic system, Vav2 and Vav3 have broader expression. Vav proteins comprise multiple domains characteristic of signal transducing proteins, including a Dbl homology domain (DH) [3–6]. The DH domain is crucial for Vav proteins to function as GTP/GDP exchange factors (GEFs) and promote signaling through activation of Rho-family small GTPases such as Rac1, Cdc42 and RhoA [1–3,5,7–9]. Vav1 is a 95-kDa protein which becomes rapidly activated in T cells, e.g. by Lck-catalyzed phosphorylation of Tyr-174 in response to T-cell receptor (TCR) and CD28 stimulation [5,10–16]. By enabling the binding of GTP to, and thus the activation of, GTPases, Vav1 and Vav2 play key roles in lymphocyte signaling, cytoskeletal rearrangements, cell migration, cytokine production, proliferation and the formation of the immunological synapse [5,17–26]. Vav1 is critical for T-cell development and T-cell-dependent immunity, whereas Vav2 appears to be more important for B-cell

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

activation [27–32]. Recent research also suggests a role of Vav proteins in innate immunity [26,33,34]. Vav proteins have been studied in Vav-knockout (ko) mice [17,19,28,35] and in Vav-deficient humans [32].

Regarding potential roles of Vav proteins in immune reactions leading to allograft rejection, there has been limited indirect evidence in that T cells are known players in graft rejection. To evaluate the role of Vav1 and Vav2 in alloreactivity and transplantation, we have studied Vav1 or Vav1/Vav2-ko mice in various immunological models. Our results show for the first time that the ko of Vav1 or Vav1/Vav2 in mice (i) leads to strongly reduced alloreactivity *in vitro* and *in vivo* and, (ii) importantly, profoundly prolongs survival times of fully mismatched heart allografts.

Animals and methods

Mice

Vav1- and Vav1/Vav2-ko mice were generated as described previously [28,36]. Vav1 ko mice were crossed onto a C57BL/6 (H-2b haplotype) background (originally received from Charles River WIGA, Sulzfeld, Germany) for at least 10 generations. Vav1/Vav2-ko mice were either crossed onto a C57BL/6 background for nine generations or maintained on a pure 129Sv background. Vav1- and Vav1/Vav2-ko and the respective wildtype (wt) mice were used as recipients of fully MHC-mismatched beige BALB/c (H-2d haplotype, Charles River WIGA) primarily vascularized cardiac grafts. Females were used as both graft donors and recipients. Several transplanted Vav1/Vav2-ko mice became obviously sick and were killed with beating cardiac allografts. The mice were fed *ad libitum* with standard diet and maintained at 21 ± 2 °C and 55 ± 10% relative humidity with a time-regulated 12-h light period. For the systemic graft-versus-host reactivity (GvH) model, female C.B-17 severe combined immune deficiency (SCID)-beige mice were supplied by Taconic, Bomholt Denmark and kept under specific pathogen-free (SPF) conditions. All mice were used at 6–10 weeks of age. For the DNP–KLH model, BALB/c female mice between 8 and 10 weeks of age were used. Mice were housed under conventional conditions in filter-top-protected cages and cared for in accordance with Swiss federal law and the NIH Principles of Laboratory Animal Care.

Flow cytometry

Splenocytes (3.8×10^5 cells/well) or whole-blood lymphocytes were activated in duplicate cultures using the 96-well round-bottomed microtiter plates (Nunclon Surface, Nunc, Denmark). Heparinized whole blood (20 μl) was dispensed into each well and ConA or phorbol 12-myristate 13-acetate (PMA)/antiCD28 antibody was added and the mixture (200 μl final volume) was then incubated at 37 °C in 5% CO_2. Samples were washed once with Cell-Wash (BD Biosciences, Basel, Switzerland). The tubes were centrifuged at 450 *g* for 5-min, supernatants discarded and the cell pellet resuspended in the remaining 100 μl buffer. Nonspecific binding was prevented by incubation with heat aggregated mouse IgG for 30 min. Fluorescent-labeled antibodies in wash buffer were added in a total volume of 20 μl, mixed and incubated for further 30 min in the dark at room temperature. Samples were stained with phycoerythrin (PE)-labeled anti-mouse CD3 monoclonal antibody (mAb) (final concentration 1 μg/ml; BD Biosciences), allophycocyanin (APC)-labeled anti-mouse CD4 or peridinin–chlorophyll–protein (PerCP)-labeled anti-mouse CD8 mAb (0.5 μg/ml; BD Biosciences) and fluorescein isothiocyanate-labeled anti-mouse CD69 mAb. Erythrocytes were lysed, cells were washed and fixed with 200 μl 1:10 diluted CellFix (BD Biosciences). Data were acquired on a FACScalibur flow cytometer (BD Biosciences) using CellQuest Plus software (BD Biosciences). Lymphocytes were gated in the forward scatter/right-angle scatter dot blot and further analyzed for expression of subset and activation markers. Data were calculated from dot blots or histograms as percentage of cells stained positively for activation markers within the target population.

Mixed lymphocyte reaction

T cells from Vav1-ko and Vav1/Vav2-ko C57BL/6 mice (H-2b) were isolated and tested in a mixed lymphocyte reaction (MLR) using irradiated BALB/c splenocytes (H-2d) as allogeneic stimulus. Single cell suspensions of splenocytes from individual Vav-ko and wt C57BL/6 mice were prepared by passage through a 70-μm nylon cell strainer (BD Biosciences) in HBSS (Invitrogen AG, Basel, Switzerland). Cells were washed twice with HBSS and resuspended at a concentration of 1×10^8 cells/ml in running buffer [PBS (Invitrogen), 0.5% bovine serum albumin (BSA) (Fluka, Buchs, Switzerland), 2 mM EDTA (Fluka)]. Purification of T cells was performed with a mouse pan T-cell isolation kit according to the manufacturer instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, non-T cells were magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies (mAbs) as primary labeling reagent and anti-biotin mAbs conjugated to MicroBeads as secondary labeling reagent. Magnetically labeled non-T cells were separated from unlabeled T cells using an AutoMACS separator (Miltenyi). T cells passing through the column were adjusted to a concentration of 4×10^6/ml in complete RPMI-1640 assay medium (Invitrogen) containing 10% heat inactivated fetal calf serum, 100 U/ml penicillin,

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

100 μg/ml streptomycin (Invitrogen) and 50 μM 2-mercaptoethanol (Fluka). The purity of the T cells was analyzed by cytometric measurement of CD3-positive cells and was usually 70–98%.

The one-way MLR was performed in 96-well flat bottom plates (Costar, Bodenheim, Germany) in 200 μl complete assay medium/well with purified T cells from Vav-ko or wt C57BL/6 mice and irradiated splenocytes from BALB/c mice as stimulator cells. Cell concentrations were 1×10^5, 2×10^5 and 4×10^5 cells/well for responder cells and 2×10^5, 4×10^5 and 8×10^5 cells/well for stimulator cells. Cultures were incubated for 4 days at 37 °C in 5% CO_2. One μCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added to each well and the plates were incubated for additional 5 h. Cells were subsequently harvested (Betaplate™ 96-well harvester, Wallac, Perkin-Elmer, Schwerzenbach, Switzerland) on filter paper Filtermat A (Wallac), which was processed for radioactivity counting in liquid scintillation counter [Beta Plate Scint in a Betaplate™ Counter (Wallac)]. Mean values and SD of triplicate cultures of the different combinations are shown as histograms. In cases where available cell numbers from individual mice were limited values of single cultures are shown.

DNP–KLH model

The antigen chosen for immunization was 2,4-dinitrophenyl (DNP) (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), conjugated to keyhole limpet hemocyanin (KLH) (Calbiochem, Läufelfingen, Switzerland) at a ratio of 20 DNP/KLH (per 100 kDa). Antigen was adsorbed to colloidal aluminum hydroxide (Alu-Gel-S, SERVA, Heidelberg, Germany) and injected intraperitoneally in mice on day 0 at 30 μg/mouse. For the serum antibody analysis, mice were bled from the tail vein on day 8, and serum was extracted after clotting for 6 h and stored at −20 °C. The levels of anti-DNP-IgM and anti-DNP-IgG (as well as the overall levels of IgM and IgG) were determined. Elisa plates (Greiner 655081, Frickenhausen, Germany) were coated with BSA–DNP (3 μg/ml), anti-IgM or anti-IgG, diluted in PBS, by overnight incubation at 4 °C. After removal of BSA–DNP solution residual, protein-binding sites were blocked with 2% BSA in PBS for 1 h at room temperature. Plates were washed thrice with PBS containing 0.05% Tween 20. This washing procedure was applied between each step. Serial threefold dilutions of the sera were performed in 2% BSA–PBS and plates incubated overnight at 4 °C. Bound IgM and IgG protein were measured using biotin-labeled goat anti-mouse IgM or IgG antibody (No. 1020-08/1030-08; Southern Biotechnology Associates, Birmingham, AL, USA) and streptavidine-conjugated alkaline phosphatase (E2636; Sigma). Enzyme activity was determined after the addition of substrate *p*-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine, pH 9.8, for 30 min at room temperature. The reaction was stopped with 1 N NaOH and quantitated colorimetrically. Five mice per experimental group were used. The serum samples from each mouse were analyzed individually to calculate mean and SD.

Systemic GvH

Single cell suspensions were prepared from spleens of Vav1- or Vav1/Vav2-ko mice and wt littermate controls. After red blood cell lysis with ACK buffer (Sigma-Aldrich), cells were labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min at 37 °C. SCID-beige recipient mice were injected i.v. with 20×10^6 unfractionated wt splenocytes or $40–60 \times 10^6$ spleen cells from Vav1- and Vav1/2 double-ko donors, respectively, to transfer 7×10^6 T cells (as determined by anti-CD3 staining). Four days after transfer, cell suspensions were prepared from individual SCID recipient spleens and T-cell recovery analyzed by four-color flow cytometry, CFSE, anti-CD4-PE, anti-CD8-PerCP and anti CD3-APC (all antibodies were purchased from BD Pharmingen, Basel, Switzerland). Flow cytometry data were acquired on a FACScalibur (BD Biosciences) using CellQuest software. Data were analyzed with FlowJo software (Treestar, San Carlos, CA, USA).

Estimates of $CD4^+$ and $CD8^+$ T-cell numbers per recipient spleen were calculated as the product of the total number of viable spleen cells (hemocytometer count, trypan blue exclusion) and the percentage of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ spleen cells within the live lymphocyte forward/side scatter gate. The percentage of $CD4^+$ or $CD8^+$ T cells that had undergone a certain number of cell cycles were derived from marker settings on CFSE histograms. For cell cycle distribution plots, the arithmetic means and SD of all individual data per recipient group are shown.

Cardiac allotransplantation

Heterotopic heart transplantation was performed as described by Corry *et al.* [37] using aseptic surgery techniques. Briefly, animals were anesthetized using isoflurane. Following heparinization of the donor mouse, the chest was opened and the heart rapidly cooled with ice cold saline. The aorta and pulmonary artery were ligated and divided and the donor heart was stored in ice cold saline. The recipient was prepared by dissection and cross-clamping of the infra-renal abdominal aorta and vena cava. The graft was implanted with end-to-side anastomoses between the donor right brachiocephalic trunk

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

and the recipient aorta and the donor right pulmonary artery to the recipient vena cava. Grafts were monitored by daily palpation and were considered rejected upon cessation of palpable ventricular contractions.

Animals were randomly assigned to groups. *Study A* ($n = 5$ per group): (i) BALB/c-to-Vav1 ko (C57BL/6), (ii) BALB/c-to-Vav1 wt (C57BL/6); *study B*: (i) BALB/c-to-Vav1 ko (C57BL/6) ($n = 8$), and (ii) BALB/c-to-BALB/c as syngeneic control ($n = 3$); *study C* ($n = 5$/group): (i) BALB/c-to-Vav1/Vav2 ko (C57BL/6), (ii) BALB/c-to-Vav1/Vav2 wt (C57BL/6), (iii) BALB/c-to-Vav1/Vav2 ko (129Sv) and (iv) BALB/c-to-Vav1/Vav2 wt (129Sv). During autopsy of long-term survivors, whole blood was sampled to perform MLR studies as described above.

Histopathology and immunohistochemistry

Cardiac allografts of study A were cut longitudinally and fixed in 4% buffered formalin together with the native heart for histological evaluation. In the other studies, cardiac allografts were cut transversally to enable morphometric analysis of vascular remodeling. Fixed tissues were processed and embedded in paraffin according to standard procedures. Three-micrometer-thick sections were stained with hematoxylin and eosin and Van Gieson for elastic fibers for light microscopic examination by an observer blinded to the identity of groups. Acute and chronic rejections were graded on scale of 0 (no rejection or no vascular intimal thickening) to 3 (severe acute cellular rejection or severe vascular intimal thickening) [38].

In addition, severity of vascular remodeling was quantified by computer-assisted image analysis using a Zeiss Image Analysis system KS400 (Carl Zeiss, Munich, Germany). Vascular remodeling is reported as the neointimal index (NI), which was analyzed on two sections per graft (study B and study C) taken always from the same relative levels of the graft ventricle (at 1/3 and 2/3 from the apex toward the atria). In study A, NI was calculated from one longitudinal section. Vessels with a diameter >35 μm from each graft were measured by computer-assisted image analysis. The NI was calculated as follows: NI = area defined by the internal elastic lamina minus area of the lumen/total area defined by the internal elastic lamina × 100. Individual measurements per each graft were averaged (mean ± SEM).

Cardiac allografts from Vav1-ko mice with survival times >100 days ($n = 12$) were analyzed immunohistochemically. Standard procedures were applied using mAbs anti-alpha smooth muscle actin (αSMA, clone 1A4, Dako-Cytomation, Glostrup, Denmark), anti-CD3 (clone CD3-12, Serotec Ltd, Oxford, UK), anti-CD45R (clone RA3-6B2, Serotec Ltd) and anti-F4/80 (clone CI:A3-1, Serotec Ltd) and the streptavidin–biotin–peroxidase complex technique. Spleen tissue served as positive control sample. Negative immunohistochemical staining controls were obtained by replacing the primary antibodies with antibody isotype controls (Zymed Laboratories, Inc., San Francisco, CA, USA).

Results

Inhibition of ConA- or PMA/aCD28-induced T-cell stimulation in whole blood

Previous studies demonstrated impaired activation of lymphocytes derived from Vav1-ko animals [17]. Here we stimulated lymphocytes in whole blood, obtained from wt and Vav-ko mice, with either ConA or a combination of PMA/aCD28 and measured upregulation of CD69 which is an early activation marker for T and B cells and represents an integral part of the activation process (Table 1). Using ConA stimulation, it was shown that the expression of CD69 in Vav1-ko $CD4^+$, $CD8^+$ and $B220^+$ cells was decreased by 81%, 89% and 74%, respectively when compared with $CD4^+$, $CD8^+$ and $B220^+$ wt controls. Surprisingly, Vav1/Vav2-ko lymphocytes showed a higher level of CD69 expression in response to ConA than the respective Vav1-ko lymphocyte subsets, but still less than the respective wt controls.

Table 1. CD69 expression (% positive cells) on lymphocyte subsets in whole blood stimulated by ConA or PMA/aCD28. Blood was collected and stimulated *in vitro* by ConA or PMA + aCD28 for 16 h. CD69 expression was measured by flow cytometry.

		ConA		PMA + aCD28	
Mouse	Cells	wt ($n = 5$)	ko	wt ($n = 5$)	ko
Vav1	$CD4^+$	36 ± 4	7 ± 1 ($n = 6$)	72 ± 8	53 ± 1 ($n = 6$)
	$CD8^+$	61 ± 3	7 ± 1 ($n = 6$)	61 ± 12	41 ± 7 ($n = 6$)
	$B220^+$	69 ± 7	18 ± 2 ($n = 6$)	50 ± 12	30 ± 6 ($n = 6$)
Vav1/Vav2	$CD4^+$	57 ± 4	22 ± 2 ($n = 7$)	85 ± 3	70 ± 4 ($n = 7$)
	$CD8^+$	70 ± 4	56 ± 6 ($n = 7$)	89 ± 3	78 ± 4 ($n = 7$)
	$B220^+$	60 ± 2	57 ± 6 ($n = 7$)	47 ± 6	84 ± 3 ($n = 7$)

Means ± SE of percentage values ($n = 5$–7).
wt, wildtype; ko, knockout.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

Using the PMA/aCD28 protocol, it was shown that the absence of Vav1 or both Vav1/Vav2 led to a small inhibition of lymphocyte activation, with the exception Vav1/Vav2-ko B220 cells where an activation (+79%) was noticed (Table 1). Thus, these lymphocyte activation studies in whole-blood cultures show that the lack of Vav proteins leads to impairment of T- and B-cell activation dependent on Vav isoform, cell type and stimulation protocol.

Inhibition of alloantigen-driven T-cell proliferation

Mixed lymphocyte reaction assays were performed to explore whether alloantigen-driven proliferation of lymphocytes is mediated by Vav1-dependent signaling. Splenic T cells isolated from Vav1-ko C57BL/6 mice or wild-type controls on a C57BL/6 background were stimulated with irradiated BALB/c spleen cells. Vav1-ko T cells showed a markedly reduced proliferation compared with wt T cells (Fig. 1a). To characterize the alloresponsiveness of Vav1/Vav2-ko T cells, an analogous MLR study was performed with purified splenic T cells from Vav1/Vav2-ko mice. As shown in Fig. 1c, T cells lacking Vav1 and Vav2 largely failed to proliferate in response to irradiated BALB/c spleen cells. To explore whether the allogeneic responsiveness of Vav-ko mice changes following allotransplantation, splenic T cells purified from Vav1- or Vav1/Vav2-ko heart allograft recipient mice 100 days after transplantation were co-cultured with BALB/c spleen cells. Essentially, no alloreactivity was observed (Fig. 1b,d). In summary, these MLR studies demonstrate a central role of Vav proteins in promoting allo-specific T-cell proliferation *in vitro*.

Deficiency in Vav1 or Vav1/Vav2 inhibits the alloantigen-driven expansion of T cells *in vivo*

We tested whether T cells from Vav1-ko and Vav1/Vav2-ko mice are capable of normal alloantigen-driven cell cycle progression in a murine systemic GvH model. CFSE-labeled spleen cells from mutant and wt mice (C57BL/6 background) were transferred into SCID mice (BALB/c background). Four days later, total spleen cell numbers and numbers of T cells retrieved from recipient spleens were determined and their CFSE staining intensity pattern analyzed. A generally reduced cellularity of

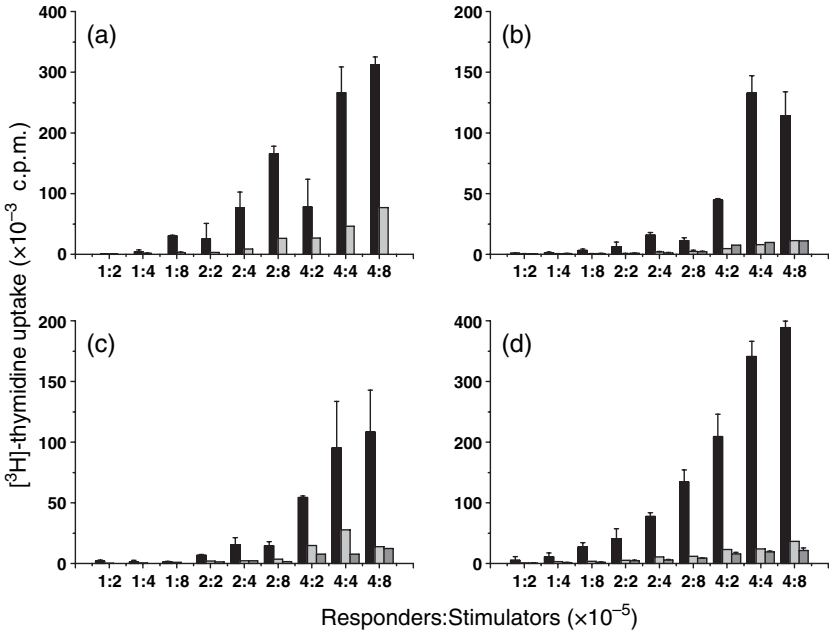

Figure 1 Effect of Vav1 and Vav1/Vav2 deficiency in T cells on their allogeneic responsiveness in an *in vitro* mixed lymphocyte reaction. Proliferation was measured by ^{3}H-thymidine incorporation. Splenic T cells from Vav1-ko C57BL/6 mice or wildtype (wt) littermates were co-cultured with irradiated BALB/c spleen cells (a, b). Vav1-ko T cells were collected from naïve C57BL/6 mice (a) or heart allograft-transplanted C57BL/6 mice on day 100 post-transplantation (b). Splenic T cells from Vav1/Vav2-ko C57BL/6 or wt mice were co-cultured with irradiated BALB/c spleen cells (c, d). Vav1/Vav2-ko T cells were collected from naïve C57BL/6 mice (c) or cardiac allograft-transplanted C57BL/6 mice on day 100 post-transplantation (d). Solid black bars indicate cultures with wt responder cells; grey bars indicate cultures with knockout (ko) responder cells. Mean values and SD of triplicate cultures are shown. When the number of cells obtained from individual mice was limited, values of single cultures are shown. (a) Results obtained from one wt and one ko mouse. (b)–(d) Results obtained from one wt mouse and two ko mice.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

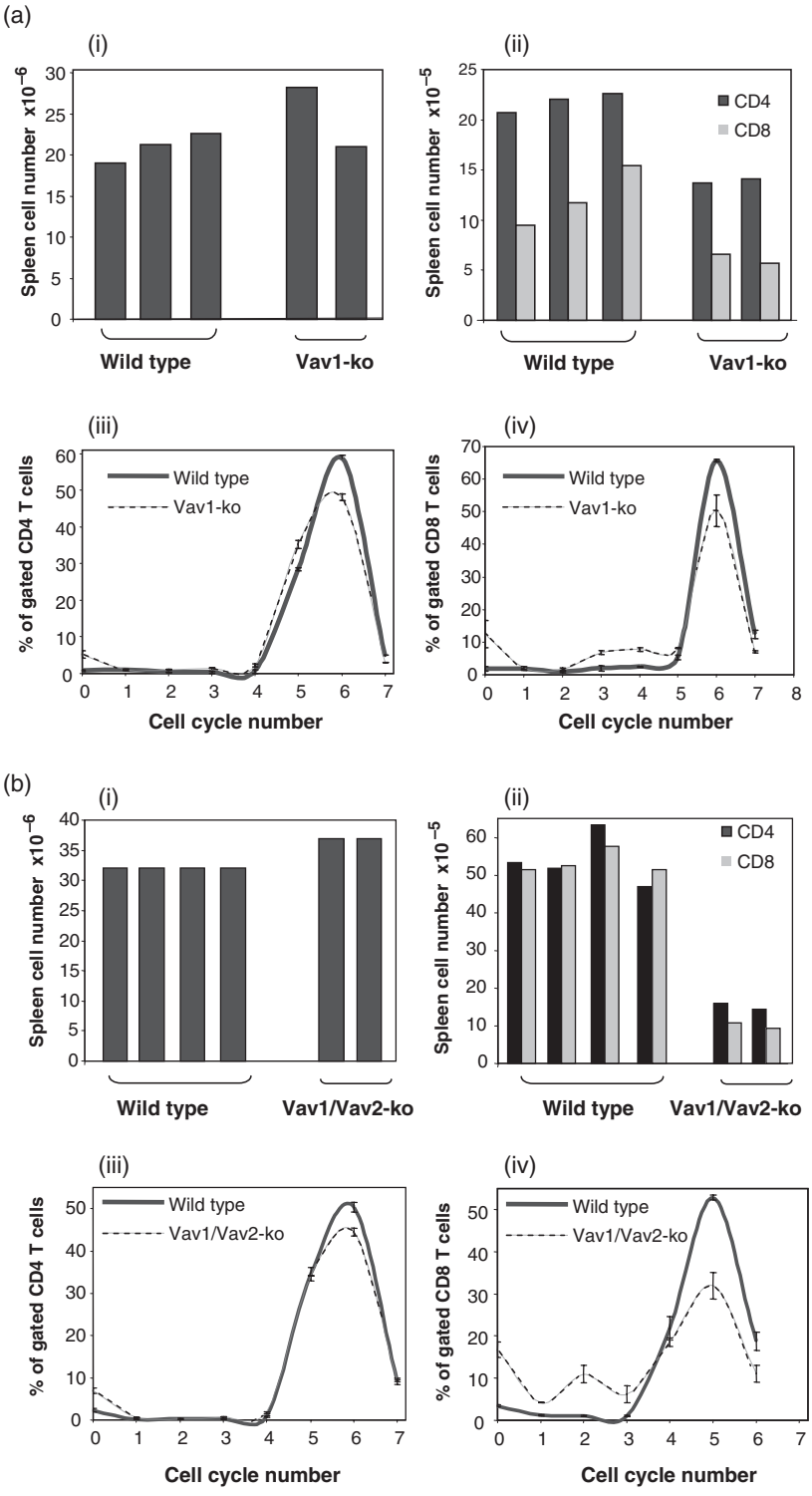

Figure 2 Vav1 and Vav1/Vav2 deficiency inhibited expansion of alloreactive T cells *in vivo*. Carboxyfluorescein diacetate succinimidyl ester-labeled donor spleen cells were injected into fully MHC-mismatched severe combined immune deficiency/beige mice. Four days after transfer, alloreactivity was assessed by T-cell recovery from recipient spleens and cell cycle analysis by FACS. (a) Vav1-ko and (b) Vav1/Vav2-ko cells.

recipient spleens was not observed after the transfer of Vav-1-ko cells when compared with the transfer of wt T cells [Fig. 2a(i)]. By contrast, the numbers of Vav1-ko T cells were reduced by about 40% for $CD4^+$ T cells and 50% for $CD8^+$ T cells [Fig. 2a(ii)] The cell cycle distribution patterns of CFSE-stained cells showed that Vav1-ko T cells still contained about 6% $CD4^+$ T cells and 12% $CD8^+$ T cells with maximal CFSE staining intensity, defined as zero cell cycles [Fig. 2a(iii,iv)]. The CFSE staining intensity of the majority of wt T cells (about

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

55% for $CD4^+$ and 65% for $CD8^+$ T cells) was consistent with six cell divisions. These proportions were about 10–20% lower for Vav1-ko T cells. Similar to the study with Vav1-ko T cells, total recipient splenic cellularity was not diminished after transfer of Vav1/Vav2-ko T cells [Fig. 2b(i)]. However, numbers of Vav1/Vav2-ko T cells were dramatically reduced by 70% and 80% for $CD4^+$ and $CD8^+$ T cells, respectively [Fig. 2b (ii)]. Cell cycle distribution patterns for $CD4^+$ and $CD8^+$ T cells were very similar compared with data obtained with Vav1-ko T cells [Fig. 2b (iii, iv)].

Thus, the systemic GvH reaction of Vav1- and Vav1/Vav2-ko responder T cells was reduced when compared with the corresponding wild-type T cells.

Vav1/Vav2 deficiency prevents T-cell-dependent antibody response to DNP–KLH

Immunization of mice with DNP–KLH enables assessment of T-cell-dependent antibody production. While the anti-DNP titer was largely suppressed in cyclosporine (CsA)-treated mice, Vav1-ko mice are fully capable of mounting a T-cell-dependent antibody response indicating that Vav1-dependent and CsA-dependent pathways are different (data not shown). By contrast, no formation of anti-DNP IgM and anti-DNP IgG antibodies was detectable in Vav1/Vav2-ko mice (Fig. 3).

Deficiency in Vav1 or Vav1/Vav2 leads to long-term cardiac allograft survival

Following the demonstration *in vitro* and *in vivo* that alloresponses are markedly reduced in T cells lacking Vav1 or Vav1/Vav2, we embarked on allotransplantation studies in Vav-ko mice. We addressed the question whether cardiac allograft survival times and histological severity of rejection were changed in Vav1- or Vav1/Vav2-ko mice.

Median survival times (MSTs) of cardiac allografts in Vav1-ko C57BL/6 mice were prolonged to >100 days without immunosuppressant treatment, while allografts of C57BL/6 wt controls were rejected with an MST of 8 days. In two independent studies, nine of 13 transplanted mice reached day 100 post-transplantation with beating allografts, while four allografts stopped beating between days 20 and 59 (Table 2). When a reverse transplantation was performed, i.e. cardiac allografts from Vav1-ko C57BL/6 mice were transplanted into BALB/c recipients, the MST was only 8 days ($n = 2$).

Transplantation studies in double ko animals were initially performed using mice on a C57BL/6 background. The genotypes were confirmed by Vav1 and Vav2 ko-specific PCR assays. In Vav1/Vav2-ko C57BL/6 mice, two of five animals reached day 100 post-transplantation with beating allografts, while the remaining three animals were killed with beating cardiac allografts caused by skin

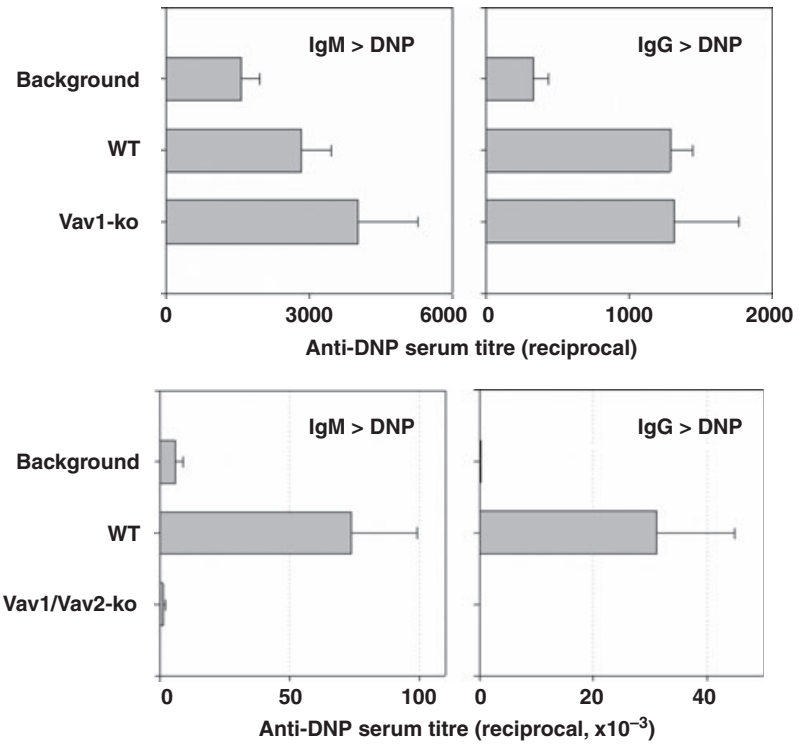

Figure 3 Anti-2,4-dinitrophenyl (DNP) antibody formation in Vav1- and Vav1/Vav2-ko mice. DNP–keyhole limpet hemocyanin was injected intraperitoneally into wildtype or mutant mice, and serum antibody levels were determined on day 8.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

Table 2. Cardiac allograft survival times in Vav1- or Vav1/Vav2-knockout (ko) mice. BALB/c cardiac allografts were transplanted into wildtype (wt) or mutant mice of C57BL/6 or 129Sv background.

Study group	Recipient of BALB/c heart	Individual graft survival	MST
A	Vav1 wt C57BL/6	7, 7, 8, 8, 8	8
A + B	Vav1-ko C57BL/6	20, 28, 50, 59, >100, >100, >100, >100, >100, >100, >100, >100, >100	>100
B	BALB/c	>100, >100, >100	>100
C	Vav1/Vav2 wt C57BL/6	9, 9, 9, 10, 25*	9
C	Vav1/Vav2-ko C57BL/6	>47†, >69†, >77†, >100, >100	>77
C	Vav1/Vav2 wt 129Sv	10, 10, 11, 11, 11	11
C	Vav1/Vav2-ko 129Sv	>39†, >40†, >75†, >76†, >100	>75

MST, median survival time.
*wt genotype verified by PCR.
†Killed with beating heart because of impaired health.

infections and body weight loss on days 47, 69 and 77 post-transplantation (Table 2). Wild-type mice rejected allografts with MST 9 days. To explore whether the genetic background played a role in the health condition seen, Vav1/Vav2-ko mice on a 129Sv background were studied. Similar to C57BL/6 mice, four of five Vav1/Vav2-ko 129Sv mice were killed before day 100 with MST >75 days due to the development of skin infections by *pasteurellaceae* and *Staphylococcus aureus*. Wt mice on 129Sv background showed graft survival times of 11 days MST. Both wt mice on C57BL/6 and 129Sv background did not show any signs of sickness.

Histopathology and vessel morphometry of cardiac allografts in Vav1- and Vav1/Vav2-ko recipients

In the control group, histological examination of all allografts of wt recipients showed acute cellular rejection with endothelialitis (grade 3). No signs of rejection were found in syngeneic transplants with two of the three isografts showing slight signs of fibrosis. Cardiac allografts of Vav1-ko recipients revealed mild acute cellular rejection and mild to severe chronic vasculopathy in all >100 days allografts ($n = 9$, Fig. 4). The NI, a measure of vessel occlusion, showed high inter-animal variability with values ranging from 25% to 93% (mean ± SD, 53.6 ± 23%). Grafts of Vav1-ko recipients, which were rejected before day 100, showed acute cellular rejection (grade 3) with different degrees of interstitial fibrosis. Immunohistochemical examination revealed mild interstitial cellular rejection composed of scattered T and B cells, and perivascular infiltrates of macrophages and mild to severe transplant vasculopathy with αSMA-positive cells and single T/B cells and macrophages.

Histological examination of cardiac allografts of Vav1/Vav2-ko recipients on both C57BL/6 and 129Sv background showed mild cellular interstitial rejection and mild transplant vasculopathy (NI measurements performed on two animals with >100 days graft survival: 32% and 25%), while the allografts of wt recipients showed severe acute cellular rejection with endothelialitis (Fig. 5).

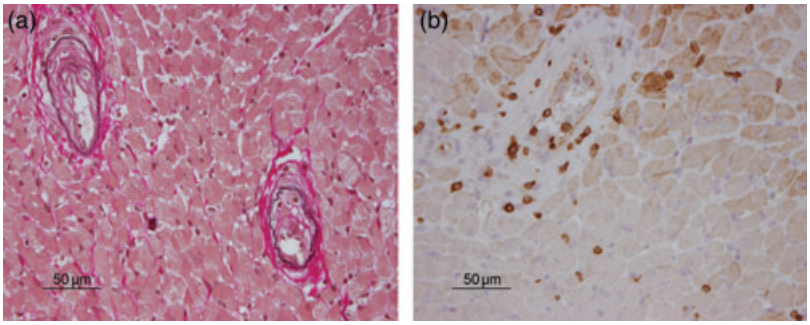

Figure 4 Histopathology of BALB/c beating cardiac allografts in Vav1-ko C57BL/6 mice removed on day 100 post-transplantation. Grafts were processed for histological examination as described under Methods. (a) Graft shows intimal thickening in intramural arteries (Van Gieson, ×400). (b) Graft shows minimal interstitial, peri-vascular and intimal infiltration of T cells (aCD3, ×400).

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

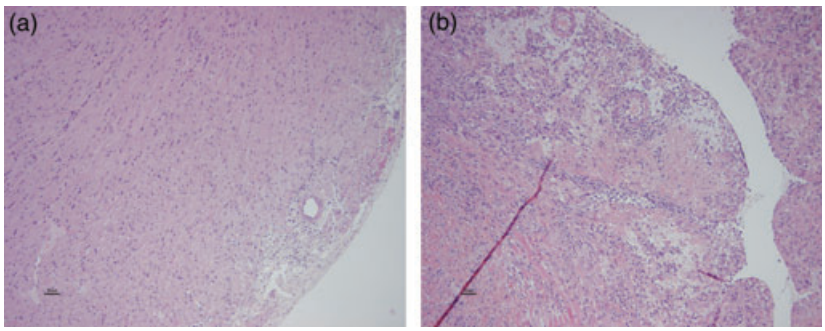

Figure 5 Histopathology of BALB/c cardiac allografts. (a) Beating allograft in Vav1/Vav2-ko C57BL/6 mice was removed on day 100 post-transplantation. The graft shows mild interstitial cellular rejection and no vascular remodeling in the intramural coronary artery shown [hematoxylin and eosin (H&E), ×100]. (b) Acutely rejecting allograft was harvested from wild-type recipient C57BL/6 mice. The graft shows the typical severe interstitial cellular rejection associated with endothelialitis (H&E, ×100).

Discussion

Using murine *in vitro* and *in vivo* models of immune responses, we showed that deficiency of Vav1 strongly suppressed alloreactivity. This led to long-term survival of fully mismatched heart allografts. Graft histology revealed low-grade inflammatory cell infiltration combined with the presence of vascular remodeling. Subsequent studies in Vav1/Vav2-ko mice showed that the double ko, while also supporting long-term graft survival, could not prevent infiltration and vascular changes. These results suggest that Vav proteins play a crucial role in alloreactivity which is in line with the key role of Vav proteins in T-cell activation by signal 1 and signal 2 [3,8,13,14]. Interestingly, while Vav1/Vav2 deficieny fully prevents T-cell-dependent antibody responses in mice, Vav1-ko mice showed a normal antibody response to ONP-KLH. Thus, although Vav1 is essential for $CD4^+$ T-cell-dependent alloreactivity, it is dispensable for a $CD4^+$ T-cell-dependent antibody response.

Vav1 is a central signal transduction protein in T cells that is activated via stimulation through the TCR and/or CD28 [8,13–16,39]. Previous work has shown that T cells lacking Vav1 or Vav1/Vav2 largely fail to respond to standard activation protocols such as stimulation with ConA or anti-CD3/anti-CD28. By contrast, full activation was still achieved when lymphocytes were exposed to PMA alone demonstrating that Vav signaling occurs upstream of PKC-dependent pathways. Our studies in whole blood cultures (Table 1) and in splenocytes derived from the same animals (not shown) confirmed the important role of Vav1 in T-cell signaling. Activation of lymphocytes with a combination of PMA and CD28 were also slightly impaired (Table 1) which is in line with the reported role of Vav proteins in signaling pathways downstream of CD28 [16,39].

We explored the impact of Vav deficiency on alloreactivity *in vitro* and *in vivo*. Our initial MLR studies using Vav1-ko splenocytes and irradiated BALB/c cells as an allogeneic stimulus suggested a strong impairment of T-cell alloreactivity due to the absence of Vav1. This initial result led to further studies as Vav-ko mice have a defect in lymphocyte maturation leading to lowered T-cell numbers in Vav1-ko mice and lowered T- and B-cell numbers in Vav1/Vav2-ko mice (28). Therefore, it was important to compensate for differences in cell number and use purified T cells from Vav1 and Vav1/Vav2 ko mice in the follow-up *in vitro* MLR studies. In these studies, we demonstrated that alloreactivity was also largely impaired when using the same number of T cells from wt and Vav1-ko or Vav1/Vav2-ko mice (Fig. 1). This result suggests that the impaired signaling previously characterized in Vav1- and Vav1/Vav2-ko T cells is mainly responsible for the suppressed alloresponse in MLR studies.

Lack of alloresponse *in vitro* was confirmed in a mouse model of systemic GvH reaction, showing markedly diminished alloantigen-driven T-cell expansion *in vivo*, in particular with Vav1/Vav2 double-ko responder T cells. CFSE staining intensity patterns revealed only slight differences, which is probably due to the late time point of data acquisition. Our previous experience using standard immunosuppressants such as CsA in this model had identified the time period of 4 days between donor T-cell transfer and retrieval to be optimal for a large 'window' in overall T-cell recovery, although earlier time points are superior for demonstrating differences in CFSE staining intensity patterns (data not shown). Due to the limited availability of Vav-ko mice, further CFSE staining studies with different time points have not been performed yet in this model. Although we have demonstrated a critical role of Vav1 in MLR and GvH, it is possible that differences in the responsiveness of Vav-ko T cells could be due to impaired thymic

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

development. To test this possibility, conditional Vav1- and Vav1/Vav2-ko phenotypes would be needed where mature lymphocytes are affected selectively.

As Vav1 plays a critical role in alloreactivity, we performed transplantation experiments using Vav-ko mice as recipients of fully mismatched heart allografts. Here, we show for the first time that the absence of Vav1 or Vav1/Vav2 in mice resulted in long-term survival times of cardiac allografts. Vav1-ko recipients of cardiac allografts reached MST >100 days in the majority of mice without any adjunct immunosuppression. These long-term surviving grafts revealed signs of mild acute cellular rejection and mild to severe chronic vasculopathy.

Analogously, Vav1/Vav2-ko recipients of cardiac allografts reached >75 days graft survival. Unfortunately, most of the animals had to be killed before grafts reached 100 days post-transplantation because of impaired health. In our hands, double ko mice kept for extended time periods seemed to be more susceptible to infectious diseases resulting in obvious sickness and hence premature termination when compared with Vav1-ko mice. This was confirmed by microbiological analysis of selected mice that had been terminated prematurely. Interestingly, we showed for the first time that Vav1/Vav2-ko mice are unable to make T-cell-dependent antibodies (Fig. 3) which are important in antiviral and antibacterial immune responses. The fact that infections occurred during the course of the study with Vav1/Vav2-ko mice but not with Vav1-ko mice hints that type and/or degree of immunodeficiency in the two mice strains is different. In this context, the recent discovery of a role of Vav1 and Vav2 in innate immune receptor signaling may be relevant [31,33] in that LPS-dependent signaling is impaired in primary Vav1/Vav2-ko B cells. It is unknown whether transplantation results obtained in Vav1/Vav2-ko mice were affected by these local infections. Based on the occurrence of infections, it was decided, in contrast to the Vav1 ko study, not to repeat the Vav1/Vav2 ko study to avoid potential undue stress to the animals. While this decreased the statistical relevance of the data for the single Vav1/Vav2-ko groups, results have to be considered in the context of the fact that very similar graft survival data were obtained with two different genetic backgrounds (C57BL/6 and 129Sv).

Like allografts in Vav1-ko recipients, long-term surviving allografts in Vav1/Vav2-ko recipients showed only mild cellular interstitial rejection. Whether development of transplant vasculopathy is reduced by additional deficiency of Vav2 and the concomitant additional impairment in antibody production needs to be addressed in a larger number of grafts, as variability of neointima formation between grafts is rather high. Studies concerning the underlying mechanism of transplant vasculopathy are ongoing.

While T-cell-dependent alloreactivity was impaired in Vav1- and Vav1/Vav2-ko mice, B-cell-based immunity is suppressed only in the double ko mice (Fig. 3). We have not assessed whether Vav1/Vav2 deficiency also directly (or only indirectly) affects B cells. Tedford *et al.* [28] demonstrated that B-cell receptor (BCR)-driven B-cell activation and proliferation are greatly impaired in Vav1/Vav2-ko B cells. Our studies show that Vav1 deficiency has no or little effect on selected B-cell functions but clearly affects $CD4^+$ T-cell function in alloreactivity without impairing $CD4^+$ T-cell helper function.

Future studies should address the role of Vav3 deficiency as well as combined deficiencies, e.g. Vav1/Vav3-ko mice, with regard to effects on alloreactivity to improve our understanding of the interplay and potential redundancies of Vav proteins [40,41]. Importantly, as suggested by our studies with Vav1/Vav2 double ko mice, the combined deficiency of selected Vav proteins may further improve graft acceptance. However, multiple Vav ko may aggravate untoward side effects due to 'over-immune-suppression'. In this context, the observation of Martinez-Gakidis [41] is relevant which showed that combined deletion of Vav1 and Vav3 leads to impaired integrin-mediated functions of neutrophils such as adhesion and phagocytosis. Similar to Vav1, Vav3 is phosphorylated and activated subsequent to TCR activation. However, while Vav3 mediates serum response element-controlled transcription, Vav1 is important for interleukin (IL)-2 production [40]. Complete deficiency of Vav1/Vav2/Vav3 was shown to lead to the development of mice without gross organic defects, but severe impairment of immune functions at both the cellular and the humoral levels [42]. In these studies, the absence of Vav3 was shown to aggravate defects in T-cell proliferation and activation induced by Vav1 deficiency alone. More insight is expected from studies in knockin mice where specific Vav functions/domains are inactivated, e.g. by single point mutations. Vav1 carrying a R422G mutation that renders the PH domain inactive and reduces GEF activity, leads to proliferation defects in $CD4^+$ T cells which, however, were less pronounced than in Vav1-ko mice [43].

In conclusion, we show for the first time that mice lacking Vav1 or Vav1/Vav2 exhibit impaired alloreactivity leading to long-term cardiac allograft survival. Interestingly, antibody responses were minimal in Vav1/Vav2 ko compared with Vav1-ko mice suggesting that reduced T-cell function is the main basis for suppressed graft rejection.

References

1. Bustelo R, Ledbetter JR, Barbacid M. Product of vav proto-oncogene defines a new class of tyrosine protein kinase substrates. *Nature* 1992; **356**: 68.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

2. Bustelo XR. Vav proteins, adaptors and cell signaling. *Oncogene* 2001; **20**: 6372.
3. Rossman KL, Der CJ, Sondek J. GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 2005; **6**: 167.
4. Palmby TR, Abe K, Der CJ. Critical role of the pleckstrin homology and cysteine-rich domains in Vav signaling and transforming activity. *J Biol Chem* 2002; **277**: 39350.
5. Turner M, Billadeau DD. Vav proteins as signal integrators for multi-subunit immune-recognition receptors. *Nat Rev Immunol* 2002; **2**: 476.
6. Zugaza JL, Lopez-Lago MA, Caloca MJ, Dosil M, Movilla N, Bustelo XR. Structural determinants for the biological activity of Vav proteins. *J Biol Chem* 2002; **277**: 45377.
7. Coso OA, Chiariello M, Yu JC, *et al.* The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 1995; **81**: 1137.
8. Swat W, Fujikawa K. The Vav family: at the crossroads of signaling pathways. *Immunol Res* 2005; **32**: 259.
9. Heo J, Thapar R, Campbell SL. Recognition and activation of Rho GTPases by Vav1 and Vav2 guanine nucleotide exchange factors. *Biochemistry* 2005; **44**: 6573.
10. Margolis B, Hu P, Katzav S, *et al.* Tyrosine phosphorylation of Vav proto-oncogene product containing SH2 domain and transcription factor motifs. *Nature* 1992; **356**: 71.
11. Crespo P, Schuebel KE, Ostrom AA, Gutkind JS, Bustelo XR. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the Vav proto-oncogene product. *Nature* 1997; **385**: 169.
12. Han J, Das B, Wei W, *et al.* Lck regulates Vav activation of members of the Rho family of GTPases. *Mol Cell Biol* 1997; **17**: 1346.
13. Tybulewicz VL, Ardouin L, Prisco A, Reynolds LF. Vav1: a key signal transducer downstream of the TCR. *Immunol Rev* 2003; **192**: 42.
14. Tybulewicz VLJ. Vav-family proteins in T-cell signalling. *Curr Opin Immunol* 2005; **17**: 267.
15. Katzav S. Vav1: an oncogene that regulates specific transcriptional activation of T cells. *Blood* 2004; **103**: 2443.
16. Blanchet F, Cardona A, Letimier FA, Hershfield MS, Acuto O. CD28 costimulatory signal induces protein arginine methylation in T cells. *J Exp Med* 2005; **202**: 371.
17. Fischer KD, Zmuidzinas A, Gardner S, Barbacid M, Bernstein A, Guidos C. Defective T-cell receptor signalling and positive selection of Vav-deficient $CD4^+$ $CD8^+$ thymocytes. *Nature* 1995; **374**: 474.
18. Tarakhovsky A, Turner M, Schaal S, *et al.* Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature* 1995; **374**: 467.
19. Holsinger LJ, Graef IA, Swat W, *et al.* Defects in actin-cap formation in Vav-deficient mice implicate an actin requirement for lymphocyte signal transduction. *Curr Biol* 1998; **8**: 563.
20. Costello PS, Walters AE, Mee PJ, *et al.* Rho-family GTP exchange factor Vav is a critical transducer of T-cell receptor signals to the calcium, ERK, and NF-κ-B pathways. *Proc Natl Acad Sci U S A* 1999; **96**: 3035.
21. Acuto O, Cantrell D. T-cell activation and the cytoskeleton. *Annu Rev Immunol* 2000; **18**: 165.
22. Wulfing C, Bauch A, Crabtree GR, Davis MM. Vav exchange factor is an essential regulator in actin dependent receptor translocation to the lymphocyte-antigen-presenting cell interface. *Proc Natl Acad Sci U S A* 2000; **97**: 10150.
23. Villalba M, Bi K, Rodriguez F, Tanaka Y, Schoenberger S, Altman A. Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J Cell Biol* 2001; **155**: 331.
24. Reynolds LF, De Bettignies C, Norton T, Beeser A, Chernoff J, Tybulewicz VLJ. Vav1 transduces T-cell receptor signals to the activation of the Ras/ERK pathway via LAT, Sos, and RasGRP1. *J Biol Chem* 2004; **279**: 18239.
25. Vicente-Manzanares M, Cruz-Adalia A, Martin-Cofreces NB, *et al.* Control of lymphocyte shape and the chemotactic response by the GTP exchange factor Vav. *Blood* 2005; **105**: 3026.
26. Wells CM, Bhavsar PJ, Evans IR, *et al.* Vav1 and Vav2 play different roles in macrophage migration and cytoskeletal organization. *Exp Cell Res* 2005; **310**: 303.
27. Billadeau DD, Mackie SM, Schoon RA, Leibson PJ. The Rho family guanine nucleotide exchange factor Vav-2 regulates the development of cell-mediated cytotoxicity. *J Exp Med* 2000; **192**: 381.
28. Tedford K, Nitschke L, Girkontaite I, *et al.* Compensation between Vav-1 and Vav-2 in B-cell development and antigen receptor signaling. *Nat Immunol* 2001; **2**: 548.
29. Turner M. The role of Vav proteins in B-cell responses. *Adv Exp Med Biol* 2002; **512**: 29.
30. Cella M, Fujikawa K, Tassi I, *et al.* Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J Exp Med* 2004; **200**: 817.
31. Hebeis B, Vigorito E, Kovesdi D, Turner M. Vav proteins are required for B-lymphocyte responses to LPS. *Blood* 2005; **106**: 635.
32. Paccani SR, Boncristiano M, Patrussi L, *et al.* Defective Vav expression and impaired F-actin reorganization in a subset of patients with common variable immunodeficiency characterized by T-cell defects. *Blood* 2005; **106**: 626.
33. Kurosaki T. Vav: a newcomer in innate receptor signaling. *Blood* 2005; **106**: 389.
34. Saborit-Villarroya I, Del Vale JM, Romero X, *et al.* The adaptor protein 3BP2 binds human CD244 and links this receptor to vav signaling, ERK activation, and NK cell killing. *J Immunol* 2005; **175**: 4226.
35. Tanaka Y, So T, Lebedeva S, Croft M, Altman A. Impaired IL-4 and c-Maf expression and enhanced Th1-cell development in Vav1-deficient mice. *Blood* 2005; **106**: 1286.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works

36. Fischer KD, Kong YY, Nishina H, *et al.* Vav is a regulator of cytoskeletal reorganization mediated by the T-cell receptor. *Curr Biol* 1998; **8**: 554.
37. Corry RJ, Winn HJ, Russel PS. Primarily vascularized allografts of hearts in mice. *Transplantation* 1973; **16**: 343.
38. Sarris GE, Mitchell RS, Billigham ME, Glasson JR, Cahill PD, Miller DC. Inhibition of accelerated cardiac allograft arteriosclerosis by fish oil. *J Thorac Cardiovasc Surg* 1989; **97**: 841.
39. Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signaling. *Nat Rev Immunol* 2003; **3**: 939.
40. Zakaria S, Gomez TS, Savoy DN, *et al.* Differential regulation of TCR-mediated gene transcription by Vav family members. *J Exp Med* 2004; **199**: 429.
41. Martinez Gakidis MA, Cullere X, Olson T, *et al.* Vav GEFs are required for beta2 integrin-dependent functions of neutrophils. *J Cell Biol* 2004; **166**: 273.
42. Fujikawa K, Miletic AV, Alt FW, *et al.* Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cell. *J Exp Med* 2003; **198**: 1595.
43. Prisco A, Vanes L, Ruf S, Trigueros C, Tybulewicz VLJ. Lineage-specific requirement for the PH domain of Vav1 in the activation of $CD4^+$ but not $CD8^+$ T cells. *Immunity* 2005; **23**: 263.

Journal compilation © 2006 European Society for Organ Transplantation
No claim to original US government works