Original Article

The distinct role of guanine nucleotide exchange factor Vav1 in Bcl-2 transcription and apoptosis inhibition in Jurkat leukemia T cells

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Aim: To investigate a novel function of proto-oncogene Vav1 in the apoptosis of human leukemia Jurkat cells.

Methods: Jurkat cells, Jurkat-derived vav1-null cells (J.Vav1) and Vav1-reconstituted J.WT cells were treated with a Fas agonist antibody, IgM clone CH11. Apoptosis was determined using propidium iodide (PI) staining, Annexin-V staining, DNA fragmentation, cleavage of caspase 3/caspase 8, and poly (ADP-ribose) polymerase (PARP). Mitochondria transmembrane potential ($\Delta\Psi_m$) was measured using DiOC₆(3) staining. Transcription and expression of the Bcl-2 family of proteins were evaluated using semi-quantitative RT-PCR and Western blot, respectively. Bcl-2 promoter activity was analyzed using luciferase reporter assays.

Results: Cells lacking Vav1 were more sensitive to Fas-mediated apoptosis than Jurkat and J.WT cells. J.Vav1 cells lost mitochondria transmembrane potential ($\Delta\Psi_m$) more rapidly upon Fas induction. These phenotypes could be rescued by re-expression of Vav1 in J.Vav1 cells. The expression of Vav1 increased the transcription of pro-survival Bcl-2. The guanine nucleotide exchange activity of Vav1 was required for enhancing Bcl-2 promoter activity, and the Vav1 downstream substrate, small GTPase Rac2, was likely involved in the control of Bcl-2 expression.

Conclusion: Vav1 protects Jurkat cells from Fas-mediated apoptosis by promoting Bcl-2 transcription through its GEF activity.

Keywords: Vav1; guanine nucleotide exchange factor; Jurkat T cells; apoptosis; Bcl-2

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Introduction

Vav1 is a member of the guanine nucleotide exchange factors (GEFs) for Rac/Rho small GTPases, and it is predominantly expressed in hematopoietic cells^[1]. The other two members of the Vav family, Vav2 and Vav3, are ubiquitously expressed^[2,3]. The functional importance of Vav1 has been demonstrated in thymocyte development, T cell activation, and tumor malignancy. Mice deficient in Vav1 exhibit decreased numbers of double positive and single positive T cells^[4,5]. This phenotype is more profound when all three Vav genes are eliminated^[6]. T cells isolated from Vav knock-out mice or genetically derived from human leukemia Jurkat T cells show obvious defects in T cell receptor (TCR)-mediated activation^[5,7]. In humans, reduced expression of Vav1 is associated with common variable immunodeficiency with defective T-cell function (T-CVID), which is characterized by hypogammaglobulinemia

due to impaired T cell function^[8]. By contrast, overexpression of Vav1 is associated with B cell chronic lymphocytic leukemia^[9]. The aberrant expression of Vav1 has also been reported in non-hematopoietic cancer malignancies, such as neuroblastoma^[10], pancreatic adenocarcinoma^[11], and melanoma^[12], indicating an indispensable role for Vav1 in cell growth and survival.

Vav proteins contain multiple structural motifs, including a Dbl homology domain for activation of Rho family GTPases, a calponin homology domain (CH), an acidic motif, a pleckstrin homology domain (PH), a cysteine-rich domain (CRD), and two SH3 domains flanking a single SH2 region^[13]. Upon T cell activation, Vav1 is rapidly tyrosine phosphorylated and uses its GEF activity on several small GTPases, including Rac1, Rac2, and Cdc42, leading to the recruitment of signaling molecules and the formation of the immune synapse^[14,15]. Vav1 also plays GEF-independent roles, such as modulating TCR-stimulated calcium flux^[16] and potentiating calcium release by association with calmodulin^[17]. Vav1 is activated upon ligation of the co-stimulatory receptor, CD28, which leads to the

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nuclear translocation of Vav1^[18]; the nuclear function of Vav1 has yet to be addressed. The structural complexity of Vav1 suggests its involvement in diverse cellular processes.

Apoptosis is an essential process in both the development of lymphocytes and the contraction phase of immune responses^[19]. Accumulating evidence suggests that Vav1 is involved in the regulation of cell apoptosis. For example, during negative selection, Vav1 promotes antigen-induced thymocyte apoptosis, and inhibitors of the actin cytoskeleton or protein kinase C (PKC) reverse the effect^[20]. In activated CD4⁺ T cells, the Vav1-Rac pathway is a critical component of TCR-induced cell death^[21]. However, Vav1 has also been reported to be a pro-survival molecule in different signaling contexts. For instance, overexpression of Vav1 counteracts CD4-mediated apoptosis of T cells by reducing mitochondrial damage and inhibiting Bax expression^[22]. Deletion of all three Vav members decreases the number of mature B cells^[23]. The pro-survival function of Vav1 is also evident in studies that treated leukemia with Vav1 antisense oligonucleotides^[24] or by blocking the Vav1-Rac signaling pathway^[25]. In addition, Vav1 expression in non-hematopoietic cells is associated with cancer malignancy^[26]. Overall, Vav1 participates in regulating cell survival, but the mechanisms of Vav1 association with the apoptotic pathway remain to be elucidated. Because Vav1 possesses promiscuous substrates and various functional motifs that overlap with Vav2 and Vav3, it is important to identify the unique role of Vav1.

Using leukemia Jurkat T cells and *vav1*-null cells (J.Vav1)^[7], we investigated the function of Vav1 in T cell apoptosis without evoking signals from the TCR and CD28. We found that the expression of Vav1 promotes Bcl-2 transcription. We further showed that Rac2 is likely to be the downstream effector of Vav1 in this signaling context.

Materials and methods

Antibodies and reagents

The anti-Vav1 and anti-Bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-ZAP70 antiserum was raised against residues 326 to 341 of human ZAP70^[7]. The anti-Fas (human, activating) IgM clone CH11 was purchased from Upstate (Lake Placid, NY, USA). The anti- α tubulin and anti-flag antibodies were purchased from Sigma (St Louis, MO, USA). The antibodies for caspase 3, caspase 8, *poly* (*ADP-ribose*) polymerase (PARP), Bcl-2, Bcl-xL, and Bim were purchased from Cell Signaling Technology (Danvers, MA, USA). PE-conjugated anti-Fas IgG was purchased from Jingmei Biotech (Beijing, China). PE-conjugated human Annexin-V recombinant protein was purchased from Bender MedSystems (Vienna, Austria). Proteinase K was obtained from Merck (Darmstadt, Germany). RNase A and the transfection reagent, polyethylenimine (PEI) were purchased from Sigma (St Louis, MO, USA). The TRIzol RNA extraction reagent was purchased from Invitrogen (Carlsbad, CA, USA). RNase-free DNase I was purchased from Promega (Madison, WI, USA). The RNA LA PCR kit (AMV) was purchased from Takara (Otsu, Shiga, Japan). *Taq* polymerase was

purchased from Tiangen (Beijing, China).

Cell lines and cell culture

Jurkat T leukemia cell line E6 was obtained from ATCC (Manassas, VA, USA). J.Vav1 cells were derived from Jurkat cells by knocking out *vav1* alleles with somatic gene targeting as described previously^[7]. The J.WT cell line was generated from a single clone of J.Vav1 cells transfected with a plasmid encoding wild-type Vav1 and selected using a drug resistance marker. HeLa and MCF7 cell lines were also from ATCC.

Jurkat, J.Vav1, and J.WT cells were grown in RPMI 1640 medium containing 10% (*v/v*) fetal bovine serum and 1% (*v/v*) penicillin/streptomycin. Cervical cancer HeLa cells were cultured in DMEM medium containing 10% (*v/v*) newborn calf serum and 1% (*v/v*) penicillin/streptomycin. Breast cancer MCF7 cells were kept in RPMI 1640 medium containing 10% (*v/v*) fetal bovine serum and 1% (*v/v*) penicillin/streptomycin. All cells were incubated at 37 °C with 5% CO₂.

Plasmid constructs

The coding region of full-length Vav1 (FL), deletion of the calponin homology domain (Del-CH), and deletion of the C-terminal SH3 domain (Del C-SH3) were subcloned into pcDNA4/HisMax vectors and designated Vav1 (FL), Vav1 (Δ CH), and Vav1 (Δ C-SH3), respectively, as described previously^[17]. The plasmids, pEF-Vav1 (L213A) and pEF-c-myc-Vav1 (Y174F), were kindly provided by Dr Altman's lab (La Jolla Institute for Allergy & Immunology, USA) and used as described previously^[7]. Flag-tagged human Vav2 and Vav3 expression plasmids were prepared by subcloning the coding region of human Vav2 and Vav3 cDNAs, respectively, into the pCMV-Tag2B vector at EcoRI-HindIII restriction sites. The Bcl-2 expression plasmid, pSG5-Bcl-2, was provided by Dr Quan CHEN (College of Life Science, Nankai University, China). The reporter plasmid, pBcl-2-Luc (Bcl-2 promoter region from ATG to -3934), was obtained from Addgene (<http://www.addgene.org/>) and originated from Dr Boxer's lab (Stanford School of Medicine, USA). The flag-tagged human Rac2 expression construct was prepared by subcloning the human Rac2 cDNA into the pCMV-Tag2B vector as a BamHI-EcoRI restriction fragment. Rac2 mutations, Rac2 (Q61L) and Rac2 (D57N), were generated by overlap PCR with the pCMV-Tag2B plasmid.

DNA transfection and luciferase reporter assay

Transfections were performed with 2×10^7 Jurkat cells or J.Vav1 cells by electroporation with a BTX Electro Square Porator, model ECM 830 (BTX Inc, San Diego, CA, USA) at 310 mv, 10 ms with 40 μ g of total plasmid DNA. For HeLa and MCF7 cells, PEI transfection was performed with 3 μ g of total plasmid DNA per 1×10^5 cells. Transfected cells were harvested 24 h later and prepared for luciferase assay. Firefly and pRL-TK-derived Renilla luciferase (RL) activities were measured according to the manufacturer's protocol with a Dual Luciferase Assay kit (Promega, Madison, WI, USA). The promoter activity was recorded as the ratio of firefly luciferase

to the RL activity and expressed as normalized relative light units (RLU). Results are presented as the mean $\pm$ standard deviation (SD) of triplicate samples. All reporter gene assays were repeated independently a minimum of three times. The expression of transfected genes was analyzed by Western blot.

Western blot analysis

Unless otherwise specified, cells (1×10^6) were collected and lysed with RIPA buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L PMSF, 1 mmol/L NaF, 1 μ g/mL leupeptin) at 4 °C. Protein concentration was determined by Bradford assay. Equal amounts of protein from each cell lysate were subjected to 10% or 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as indicated. The resolved proteins were transferred to PVDF membranes and blotted with indicated antibodies.

Flow cytometry analysis

Cells were collected, and 1×10^6 cells per mL were resuspended in 1 $\times$ PBS (pH 7.4) containing 1% FBS and incubated with PE-conjugated Annexin-V or propidium iodide (PI) in the dark for 20 min at room temperature. Cells were then washed once with 1 mL of PBS and resuspended in 500 μ L of PBS containing 1% FBS. Flow cytometric analysis was performed on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton Dickinson).

For mitochondrial transmembrane potential ($\Delta\Psi_m$) measurement, cells were treated with 10 ng/mL CH11 or left untreated for 6 h. The $\Delta\Psi_m$ indicator, DiOC₆(3) (2 μ L of a 2 μ mol/L stock solution in DMSO), was added to a 400 μ L cell suspension in PRMI-1640 medium and incubated at 37 °C for 5 min. $\Delta\Psi_m$ was analyzed by flow cytometry with excitation at 488 nm. A decrease in DiOC₆(3) fluorescence (530 $\pm$ 30 nm) indicates a loss of $\Delta\Psi_m$.

DNA fragmentation

One million cells were collected by centrifugation (room temperature, 110 $\times$ g, 5 min), washed briefly with 1 $\times$ PBS (pH 7.4) and centrifuged again. Cell pellets were then treated with 50 μ L lysis buffer (1% NP40 in 20 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 7.5), and supernatants were collected by centrifugation (room temperature, 1600 $\times$ g, 5 min). The supernatants were then sequentially incubated with RNase A (5 μ g/ μ L, 37 °C, 2 h) and proteinase K (2.5 μ g/ μ L, 58 °C, 2 h) to eliminate RNA and protein contamination. The extracted DNA was obtained by ethanol precipitation, dissolved in gel loading buffer, and separated by agarose gel electrophoresis.

RNA isolation and semi-quantitative RT PCR

Total RNA was isolated from 1×10^7 cells as indicated with TRIzol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The RNA was then resuspended in DEPC-treated TE (pH 8.0) and treated with RNase-free DNase I (Promega) for 1 h. Two micrograms of total RNA was reverse-transcribed at 42 °C using the Takara RNA LA PCR kit (AMV) with oligo dT primers. Semi-quantitative

PCR was performed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control for gene expression. The primers used for semi-quantitative PCR were as follows: Bcl-2 5'-TTCTTTGAGTTCGGTGGGGTC-3' (forward) and 5'-AGCCAGGAGAAATCAAACAGAGG-3' (reverse); HPRT 5'-TGACACTGGCAAAACAATGCA-3' (forward) and 5'-GGTCCTTTTCACCAGCAAGCT-3' (reverse). The PCR products were resolved by 1.5% agarose gel electrophoresis. The net intensity of each band was quantified using Quantity One software (BIO-RAD, Hercules, CA, USA).

Statistical analysis

Most of our results are representative of at least three independent experiments and are presented as the mean $\pm$ standard deviation (SD) of triplicate samples.

Results

Loss of Vav1 leads to high sensitivity of cells to apoptosis

Fas-mediated apoptosis is the main mechanism of T cell homeostasis. Here, we used the Fas agonist antibody, CH11, to induce apoptosis in cells in the presence or absence of Vav1 (Figure 1A). After treatment with 10 ng/mL of CH11 for 8 h, 50% of J.Vav1 cells underwent apoptosis, whereas 70%–80% of Jurkat or J.WT cells were viable based on PI analysis (Figure 1B), indicating a protective role for Vav1 against Fas-mediated cell death. To exclude possible variations in expression of Fas due to the cloning process, we stained the three cell lines with a PE conjugated Fas-specific antibody (IgG) that does not induce apoptosis. Flow cytometry analysis showed the same shift in PE fluorescence intensity (Figure 1C), indicating the same level of surface Fas molecules on these cells. One of the characteristic early events of apoptosis is the reversed location of phosphatidylserine. When treated with CH11 for 12 h, nearly 50% of J.Vav1 cells were Annexin-V positive in comparison with 30% of Jurkat or J.WT cells that were Annexin-V positive (Figure 1D). The final event of apoptosis, DNA fragmentation, was also found to be higher in J.Vav1 (Figure 1E) *versus* Jurkat and J.WT cells. The above results indicate that cells lacking Vav1 are more sensitive to apoptosis by CH11. Similar trends of cell death were also seen with other apoptotic stimuli, such as serum starvation and oxidation (Supplementary Figure 1). These data indicate that the loss of Vav1 results in cells being more sensitive to apoptotic stimuli. We also examined the cleavage of the terminal molecule in the caspase pathway, caspase 3, and its substrate, PARP, and observed an earlier cleavage and activation pattern of caspase 3/PARP in J.Vav1 cells (Figure 1F).

Fas engagement leads to the formation of the DISC (death inducing signaling complex) and the activation of caspase 8, and Vav1 could negatively regulate DISC formation by competing for actin binding sites with Ezrin, a linker between Fas and the actin cytoskeleton^[27]. Thus, we examined the kinetics of caspase 8 cleavage in the presence or absence of Vav1 upon anti-Fas CH11 treatment. We found detectable

Figure 1. Effect of Vav1 on Fas-mediated cell death. (A) Vav1 protein expression in the indicated cells was determined by immunoblot using an antibody against Vav1, and ZAP70 was used as a loading control. (B) Cells with or without Vav1 were treated with increasing concentrations of CH11 for 8 h, and percentages of live cells were determined by flow cytometry using PI staining. Data represent the mean with standard deviation of 3 experiments. (C) Expression of surface Fas was analyzed by flow cytometry with PE-conjugated anti-Fas IgG (darker lines). The pre-immune IgG was used as control (lighter lines). (D) Comparison of Fas-mediated apoptosis with or without Vav1. Cells were treated with CH11 (10 ng/mL) for 12 h and stained with Annexin-V PE. The apoptotic cells (AV⁺) were analyzed by flow cytometry, and percentages of AV⁺ cells are quantitatively displayed in the graph. (E) Cells were treated with CH11 for the indicated time, and extracted DNA was subjected to agarose gel electrophoresis. Cleavage of caspase 3, PARP (F) and caspase 8 (G) in cell lysates was examined after treatment with CH11 (10 ng/mL) for the indicated times. Tubulin was used as a loading control. (A), (C), (D), (E), (F), and (G) are representative of at least three independent experiments.

cleavage of caspase 8 1 h after treatment in J.Vav1 and J.WT cells and 2 h after treatment in Jurkat cells (Figure 1G). The decreased expression of Vav1 in J.WT compared to Jurkat cells may not provide enough Vav1 to compete with Ezrin and thus cannot block the formation of the DISC. On the other hand, the rescue of caspase 3 and PARP in J.WT (Figure 1F) also indicates that caspase 8 is not the upstream mediator of caspase 3 cleavage.

As Jurkat cells are type II cells in which the FasL-induced apoptosis is mitochondria-dependent^[28], the above data suggest that Vav1 plays an anti-apoptotic role in mitochondria-dependent apoptosis.

Vav1 maintains mitochondrial membrane integrity

The disintegration of the mitochondrial membrane marks the earliest event of apoptosis upon CH11 treatment. We determined the change in mitochondrial transmembrane

potential ($\Delta\Psi_m$) in Jurkat, J.Vav1, and J.WT cells by flow cytometry using the fluorescent dye, DiOC₆(3), as a tracer. We found that in the absence of Vav1 (J.Vav1), more than 62% of cells had lower $\Delta\Psi_m$ compared with that of Jurkat (35%) and J.WT cells (39%) (Figure 2). Thus, the integrity of the mitochondrial membrane is severely impaired in the absence of Vav1 upon treatment with CH11.

Vav1 sustains Bcl-2 transcription in T cells

T cell fate is precisely controlled by the balanced expression of the Bcl-2 family of proteins. In activated T cells, Bcl-2 levels are reduced by nearly 50%, allowing them to undergo activation-induced apoptosis in the contraction phase of T cell response^[29]. We therefore asked whether there were differences in the expression of Bcl-2-related proteins. A panel of pro-apoptotic proteins (Bax, Bim, Bmf, Puma, and Bok) and anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-

Figure 2. Effect of Vav1 on mitochondrial membrane integrity. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) in the three cell lines were detected by DiOC₆(3) staining after CH11 treatment. The percentages of cells shifting to lower fluorescence intensity are shown in the bar graph. These data are representative of three independent experiments.

1) were examined. Among these proteins, the expression of Bcl-2 was significantly reduced in J.Vav1 cells, but not in Jurkat or J.WT cells. No significant differences in expression of the other family members, such as pro-survival Bcl-xL, pro-apoptotic Bax, or BH3 only Bim, were observed (Figure 3A). This experiment demonstrated that loss of Vav1 decreased the expression of Bcl-2, and reconstitution of cells with Vav1 restored the levels of Bcl-2. Therefore, Vav1 is required for maintaining the expression of Bcl-2 in Jurkat T cells. Because the level of Bcl-2 proteins in cells is strictly regulated, the transcription of Bcl-2 was examined. Semi-quantitative RT-PCR showed that the level of Bcl-2 mRNA in J.Vav1 cells was only half of that in Jurkat or J.WT cells (Figure 3B), in agreement with the observed reduction in Bcl-2 protein expression (Figure 3A). The Bcl-2 synthesis and degradation rates were unchanged in the presence or absence of Vav1 (data not shown). Therefore, Vav1 affects the transcription of Bcl-2.

To confirm the effect of Vav1 on Bcl-2 transcription, we performed a reporter assay in which the luciferase gene was under the control of the full promoter region (from ATG to -3934) of Bcl-2^[30]. Transient expression of Vav1 increased Bcl-2 promoter activity to the level as that in Jurkat cells (Figure 3C), whereas J.Vav1 cells transfected with control empty vectors displayed only 50% of the promoter activity observed in Jurkat cells (Figure 3C), consistent with the mRNA analysis (Figure 3B). This suggests that Vav1 promotes Bcl-2 tran-

scription in Jurkat cells. In addition, Bcl-2 transcription was further enhanced in Jurkat cells overexpressing Vav1 (Figure 3C), implying a dose-dependent transcription of Bcl-2 by Vav1. In agreement with this, the ectopic expression of Vav1 or Bcl-2 could rescue J.Vav1 from CH11-induced cell death (Figure 3D), suggesting that the amount of Vav1 influences the transcription of Bcl-2.

The GEF activity of Vav1 is specifically required for maintaining Bcl-2 transcription

Vav1 contains multiple structural domains that permit its function as a GEF and a scaffold protein^[31, 32]. To explore the possible mechanisms by which Vav1 enhances the transcription of Bcl-2, we constructed a series of Vav1 mutations (Figure 4A). As previously reported, disruption of the Dbl homology domain by the L213 mutation, referred to as Vav1 (L213A), abolishes the GEF activity of Vav1. By contrast, Vav1 (Y174F) and Vav1 (Δ CH) possess constitutive GEF activity due to the unmasked DH domain^[13, 33]. Deletion of the C-terminal SH3 domain, Vav1 (Δ C-SH3), eliminates the interaction of Vav1 with many nuclear proteins^[32]. Expression of the Bcl-2 reporter gene was measured in the presence of the above Vav1 mutations. Whereas the expression of wild-type Vav1 in J.Vav1 cells increased luciferase expression by more than two-fold of that in the control sample (pcDNA4), Vav1 (Y174F) and Vav1 (Δ CH) further enhanced Bcl-2 transcription (Figure 4B). By contrast, GEF-deficient Vav1 (L213A) failed to induce Bcl-2 transcription (Figure 4B). Vav1 (Δ C-SH3) promoted Bcl-2 transcription to levels similar to wild-type Vav1 (Figure 4B), ruling out the effect of nuclear interactions of Vav1. Taken together, the mutation analyses demonstrate that the GEF activity of Vav1 is required for maintaining Bcl-2 transcription.

Vav family members (Vav1, 2, and 3) possess highly homologous DH domains and redundant GEF functions. Because of the presence of Vav2 and Vav3 in Jurkat cells, we examined their effect on Bcl-2 transcription and found that overexpressed Vav2 or Vav3 had no significant influence on Bcl-2 promoter activation (Figure 4C). Thus, among Vav family members, only Vav1 is responsible for promoting the transcription of Bcl-2 in Jurkat T cells. We further tested the effect of Vav1 expression on Bcl-2 transcription in the non-hematopoietic cancer cell lines, HeLa and MCF7, and found similar trends in Bcl-2 promoter activity (Figure 4D).

Vav1 enhances Bcl-2 transcription via the small GTPase, Rac2

Vav family proteins function as GEFs for many small GTPases with nonspecific and overlapping specificity^[14]. Vav1 can activate the GDP/GTP exchange for Rac1, Rac2, and RhoG; Vav2 is a GEF for RhoA, RhoB, and RhoG; and Vav3 preferentially activates RhoA, RhoG, and to a lesser extent, Rac1^[32]. Among these small GTPases, Rac2 is predominantly expressed in hematopoietic cells and is activated by Vav1^[34]. To investigate whether Rac2 was downstream of Vav1 in the regulation of Bcl-2 transcription, we examined cells with mutations in Rac2. The results showed that full-length Vav1 enhanced reporter activity, whereas Rac2 alone had no detectable effect

Figure 3. Expression of the Bcl-2 family of proteins in the presence or absence of Vav1. (A) Expression of indicated Bcl-2 family members was analyzed by Western blot with tubulin as the loading control. (B) Bcl-2 transcripts in Jurkat, J.Vav1, and J.WT cells were analyzed by semi-quantitative RT-PCR. The relative amount of Bcl-2 mRNA was calculated as a ratio of the control HPRT mRNA (bar graph). (C) Jurkat cells (solid bars) and J.Vav1 cells (open bars) were co-transfected with the pBcl-2-Luc reporter and pTK-RL plasmids, together with the empty vector (pcDNA4) or the indicated amount of the Vav1 plasmid. Twenty-four hours post-transfection, cells were collected, and Bcl-2 promoter activity was measured as firefly luciferase activity normalized to the Renilla luciferase activity and presented as relative light units (RLU). Bars indicate the mean $\pm$ SD from triplicate samples. The lower panel shows the expression of Vav1 protein in each sample, with tubulin as the loading control. (D) J.Vav1 cells were co-transfected with pEGFP plus control vector (pcDNA4) and the Vav1 expression vector or Bcl-2 expression plasmid. Twenty-four hours later, each transfection group was divided into two groups with one group treated with 20 ng/mL CH11 and the other left untreated for 5 h, and cells were then analyzed by flow cytometry. The percentage of live cells was obtained by dividing the percentage of GFP positive cells in the CH11-treated group by that in the untreated group. Bars indicate the mean $\pm$ SD from triplicate samples. Protein expression was determined by immunoblot with lysates from total transfected cells (lower panel). Data in (A) and (B) are representative of three independent experiments

on promoter activity and the constitutively active Rac2 (Q61L) increased Bcl-2 transcription to levels similar to Vav1 (FL) (Figure 5A). Expression of a dominant negative form of Rac2, Rac2 (D57N), further suppressed Bcl-2 promoter activity (Figure 5A) and suppressed 64% and 51% of the Bcl-2 promoter activity enhanced by Vav1 and Vav1 (Y174), respectively (Figure 5B). For comparison, we also tested the small GTPase Rac1, a major downstream effector of Vav1 during TCR activation, and the constitutively active form of Rac1 failed to increase Bcl-2 transcription (data not shown). These data indicate that Rac2 is a molecule downstream of Vav1 for Bcl-2 transcription.

Discussion

Apoptosis is important for maintenance of T cell homeostasis. Vav1 has been implicated in the regulation of T cell survival in both physiological and pathological processes^[16] in addition to its essential role in T cell activation. Here, the use of

a *vav1* genetically knocked-out cell line, J.Vav1, enabled us to address the distinct role of Vav1 in the regulation of apoptosis; this role is not compensated by the co-existing Vav2 and Vav3. In our model, Vav1 promotes Bcl-2 transcription through its GEF activity and the downstream small GTPase, Rac2. The increased Bcl-2 expression protects mitochondrial membrane integrity against apoptotic stimuli (Figure 6). Vav1 has also been reported to promote antigen-induced thymocyte apoptosis during negative selection^[20], and the Vav1-Rac pathway is a critical component for TCR-induced CD4⁺ T cell death^[21]. We speculate that Vav1 may play opposing roles in different contexts; Vav1 has a pro-survival role in quiescent T cells to maintain homeostasis and a pro-apoptotic role during T cell responses. In support of this, we found that Vav1 promoted Bcl-2 transcription in non-hematopoietic cells, which may contribute to cancer malignancy in conjunction with Vav1 overexpression.

As one of the GEFs for the Rho family of small GTPases,

Figure 4. Effect of Vav1 mutations on Bcl-2 promoter activity. (A) A schematic depiction of Vav1 and its mutations. The structural domains of Vav1 are indicated on the top line. Solid bars indicate the mutated forms of Vav1, and point mutations were indicated with stars. The plasmids, pBcl-2-Luc and control reporter pTK-RL, were transfected into J.Vav1 cells (B), Jurkat cells (C), or non-hematopoietic HeLa or MCF7 cells (D) with control pcDNA4 or protein expression plasmids as indicated. Luciferase activity in each sample was measured and presented either as RLU (B) or as fold induction over the control group (C and D). The results show the mean±SD from triplicate samples. The expression of transfected proteins was analyzed by immunoblot with tubulin as the loading control.

the function of Vav1 is important in TCR-mediated signal transduction and actin polymerization^[32]. There are GEF-dependent and GEF-independent activities modulating cellular signaling. As all three Vav proteins are present in T cells, the high structural similarity and overlapping functions of Vav proteins suggest that they may compensate for the loss of another. The significance of Vav1 GEF activity in T cell activation has been recently challenged because TCR-mediated actin polymerization and Rac1 activation were rescued by introduction of a GEF-inactive Vav1 in J.Vav1 or primary T cells lacking all Vav members^[35]. Thus, the GEF function of Vav1 is not required for T cell activation. This raises the question of the genuine cellular function of the Vav1 GEF. Our data demonstrated the requirement of the GEF activity of Vav1, but not Vav2 or Vav3, in inducing Bcl-2 transcription (Figure 3C), and neither endogenous nor transiently expressed Vav2 or Vav3 affected Bcl-2 transcription in Jurkat cells (Figure 4C). These results suggest that one of the functions of the Vav1 GEF lies in the activation of its downstream GTPase(s) to induce Bcl-2 transcription.

Small GTPases are critical for regulating cell survival, and one of their functional mechanisms is to induce the transcription of the Bcl-2 family of proteins. We identified Rac2 as the likely downstream effector of Vav1 in the context of Bcl-2 transcription rather than the commonly recognized Rac1 in T cell activation. Rac2 has been reported to participate in apop-

toxis. Mast cells from Rac2 knock-out mice are deficient in growth factor-induced survival and have increased expression of Bad and decreased expression of Bcl-xL at basal levels^[36]. Because Vav1 and Rac2 are both predominantly expressed in hematopoietic cells, it is very likely that the transcriptional control of Bcl-2 by Vav1-Rac2 is also specific for hematopoietic cells. However, we cannot rule out the participation of other Vav1 substrates in this context.

The involvement of Vav1 in transcription has been suggested in previous studies by its nuclear localization and binding to nuclear proteins using its C-SH3 domain^[37-40]. The nuclear function of Vav1 has been further emphasized by its association with DNA as a transcriptional complex^[41]. We tested Bcl-2 transcription in the presence of different mutations of Vav1 using a reporter assay and found that the C-SH3 domain had no obvious effect on the promoter activity of Bcl-2 (Figure 4B). Evidence from *vav* triple knock-out mice has indicated that reduced NFκB activity may be responsible for the decrease in Bcl-2 expression. Indeed, we previously showed decreased NFκB activity in the absence of Vav1 (J.Vav1), and reconstituted expression of Vav1 could counteract this deficiency^[7]. Furthermore, caspase 3 has been found to cleave Vav1 at the DLYD¹⁶¹C site during apoptosis, and the cleaved Vav1 displayed a severely impaired ability to activate NFκB and AP-1^[42]. Therefore, the transcriptional control of Bcl-2 by Vav1 does not occur directly via its predicted transcriptional activity, rather it involves signaling pathways that control the

Figure 5. Effect of small GTPase, Rac2, on Bcl-2 promoter activity. (A) J.Vav1 cells were co-transfected with pBcl-2-Luc reporter constructs plus an empty vector (pcDNA4), a plasmid encoding full-length Vav1 (FL), or flag-tagged Rac2 variants (WT: wild-type; constitutively active Q61L; or dominant negative D57N). Luciferase activity of each transfection was measured and presented as RLU. The results are shown as the mean $\pm$ SD from triplicate samples. (B) J.Vav1 cells were transiently transfected with reporter constructs (as above) together with wild-type Vav1 (FL) or constitutively active Vav1 (Y174F), in combination with vehicle plasmid (pcDNA4) or dominant negative Rac2 (D57N). The inhibition of Rac2 (D57N) (solid bars) on Bcl-2 promoter activity is presented as the percentage of vehicle control (open bars). Bar graph shows the mean $\pm$ SD from triplicate experiments. The expression of transfected Vav1 and Rac2 were analyzed by immunoblot using α -Vav1 and α -flag antibodies with tubulin as the loading control (lower panel).

Figure 6. A model for Vav1 function in apoptosis.

balance between cell survival and cell death.

In conclusion, our results showed that the loss of Vav1 led to a higher sensitivity of Jurkat cells to the induction of apoptosis, including via Fas activation. We further found that mRNA levels of Bcl-2 were significantly reduced in J.Vav1 cells. The GEF activity of Vav1 was essential to the transcription of Bcl-

2, probably via the Vav1 substrate, Rac2. The transcriptional control of Bcl-2 was exclusive to Vav1 despite the presence of the other Vav family proteins, Vav2 and Vav3. This finding suggests that Vav1, in addition to its importance in TCR-mediated T cell activation, plays a distinct role in controlling the transcription of Bcl-2 and thus protects T cells from apoptosis. Unveiling these mechanisms helps to better understand the homeostatic control of lymphocytes. As Vav1 is specifically expressed in hematopoietic cells, control of Vav1 expression and its GEF activity may be a potential therapeutic strategy for the treatment of T-CVID and lymphoma.

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Author contribution

Jie YIN and Dr You-jia CAO initiated the project, Jie YIN conducted most of the experiments, Ya-juan WAN and Ming-juan DU helped on the plasmid constructions and some biochemistry assays, Shi-yang Li and Xing-long ZHOU performed the flow cytometry for apoptotic analysis, Dr Cui-zhu ZHANG provided technical support, and Dr You-jia CAO and Jie YIN wrote the manuscript.

Supplementary information

Supplementary figure is available at Acta Pharmacologica Sinica website of NPG.

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