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Regulating Vav1 phosphorylation by the SHP-1 tyrosine phosphatase is a fine-tuning mechanism for the negative regulation of DISC formation and Fas-mediated cell death signaling

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The actin cytoskeleton association is required for caspase 8-independent Fas/CD95 receptor internalization, a critical step for an optimal death-inducing signaling complex formation along the endocytic pathway, leading to efficient activation of the caspase cascade and, ultimately, cell death. However, the way in which this initiation phase of Fas receptor signaling is regulated is still unknown. We report herein that, in B cells, upon Fas engagement, the tyrosine phosphatase SHP-1-regulated Vav dephosphorylation, by downmodulating the Fas–ezrin–actin linkage is a fine-tune switch-off mechanism that the cell uses as a way to terminate the receptor internalization, controlling therefore the time and extent of the DISC formation and cell death.
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Among apoptotic pathways, the one triggered by the death receptor (DR) Fas (CD95/Apo-1/TNFSFR6), a member of the tumor necrosis factor superfamily receptor (TNFSFR), upon engagement by its ligand, Fas ligand (FasL), is one of the most widely studied.[1]

This receptor–ligand system is particularly important for the maintenance of lymphocyte homeostasis. Upon engagement by FasL, Fas rapidly recruits the adapter molecule FADD and caspase 8 proenzyme, via homologous death domain and death effector domain (DED) interactions, forming the death-inducing signaling complex (DISC). This leads to caspase activation and ultimately apoptosis.[2,3] Despite the wealth of information on events downstream of DISC formation, little is known about the events necessary to generate and regulate the early formation of this complex.

During recent years, several studies were carried out to dissect the molecular mechanisms that govern the fate of Fas receptors at the cell surface upon FasL engagement before DISC formation. Two main features were determined: (i) the importance of Fas association (both constitutive and induced by FasL) with membrane microdomains or rafts;[4–6] (ii) a clathrin-dependent internalization of the receptor as an additional requirement for Fas-mediated cell death.[7,8] Very recently, a molecular link between these two requirements was identified: We showed that post-translational modification of Fas by palmitoylation is a key step in the targeting of the receptor to the membrane microdomains. This event allows the indispensable connection between Fas and the actin cytoskeleton to occurs, via the association of Fas with ezrin upon FasL engagement.[9] Importantly, we demonstrated that this association is critical for the caspase 8-independent receptor internalization, which we propose to be indispensable for the intracellular amplification of Fas signaling brought about by extensive DISC formation leading to efficient activation of the caspase cascade and, ultimately, cell death.[9] Nevertheless, while Fas internalization within the rafts appears essential for Fas-mediated cell death, the precise mechanism regulating this process is still largely unknown.

The interaction between FasL and Fas plays an essential role in the control of T- and B-cell activity and maintenance of immunological tolerance. Lack of function mutations in the Fas receptor (e.g., in *lpr* mice or in humans with autoimmune lymphoproliferative syndrome) or FasL (*gld* mice) can be associated with immune dysfunction. These include autoantibody production, accumulation of lymphoid cells displaying an activated phenotype leading to lymphadenopathy and splenomegaly, and increased development of B lymphomas.[10] Recently, it was shown that the SH2-containing PTP1 (SHP-1) protein, one of the most important negative regulators of B-cell activation, was associated with DRs of the TNFSFR, including Fas.[11] However, the precise function of this phosphatase in the Fas-mediated cell death pathway is still unknown. SHP-1 is expressed primarily in hematopoietic cells and usually functions as a negative regulator, dephosphorylating certain molecules involved in the early signal transduction.[12] Studies of B cells from SHP-1-deficient motheaten mice have shown that SHP-1 plays an important role in B-cell differentiation, proliferation and survival.[13]

In the context of cell death signaling regulation, both positive and negative effects of SHP-1 have been

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Keywords: Fas; signaling; Vav; SHP-1
Abbreviations: DED, death effector domain; DISC, death-inducing signaling complex; DR, death receptor; FasL, Fas ligand; LtnA, latrunculin A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHP-1, SH2-containing PTP1 protein; TNFSFR, tumor necrosis factor superfamily receptor
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reported,[14,15] and apart from a single report claiming that the association of Fas with SHP-1 is involved in the downregulation of the IL-3 receptor-mediated survival signal in neutrophils,[11] no corresponding molecular events have thus far been delineated.

Several SHP-1 substrates have been identified, including CD72, Syc, BLN, SLP-76, lck, jak3 and Vav1.[16] Vav1 dephosphorylation by SHP-1 is reported to be critical in the inhibition of natural killer cell cytotoxicity due to its ability to block the actin-dependent activation signal.[17] In addition, both actin[18] and myosin[19] have been identified as direct SHP-1 substrates in B cells. Cells transfected with a catalytically inactive form of SHP-1 displayed more prolonged actin polymerization.

Vav1, a member of the Dbl superfamily of Rho/Rac guanine nucleotide exchange factors that is mainly expressed in the hematopoietic lineage, is well known as a central regulator of actin cytoskeletal rearrangements during cell activation.[20] Both the guanine nucleotide exchange factor activity and the adaptor functions of Vav are tightly regulated by tyrosine phosphorylation of the protein.[21] Conflicting reports have described both activation and inhibition of cell death by Vav.[22,23] However, very little data on the functions of Vav in Fas-mediated signaling are available, except a report describing a higher level of Vav tyrosine phosphorylation in the Fas-deficient MRL/Mp-*lpr*/*lpr* mice.[24]

The importance of cytoskeletal rearrangement in Fas-mediated cell death is well known, while DISC formation is dependent on actin filaments.[25,26] However, the way in which this initiation phase of Fas receptor signaling is regulated has remained unknown. Here, we report that SHP-1 blocks the actin-dependent activation signal through the regulation of the Vav protein dephosphorylation, thereby inhibiting Fas internalization and subsequent DISC formation and cell death signaling.

Results

The absence of SHP-1 increases Fas-induced cell death. SHP-1 has been described either as a positive or a negative regulator of different cell death pathways. Its role in Fas-mediated cell death is rather controversial. Whereas the SHP-1–Fas association has been reported to elicit an inhibitory effect on the IL-3-mediated survival pathway, without any direct role in Fas-induced cell death,[11] the interaction of SHP-1 with other non-apoptotic receptors has been demonstrated to act as an immediate regulator of Fas-mediated cell death.[14] To analyze the role of SHP-1 in Fas signaling, we established A20 mouse B cells stably expressing SHP-1 shRNAi, in which expression of SHP-1 was efficiently inhibited when compared to cells expressing control shRNAi. The expression of Fas receptor at the cell surface remained unaffected (Figure 1a). We next compared the sensitivity of these cells to FasL-induced cell death. The SHP-1 shRNAi A20 cells exhibited a more rapid and efficient Fas-mediated cell death compared to the A20 cells expressing control shRNAi (Figure 1b). The same result was obtained when cell death was triggered by rhFasL or by coculture with membrane-bound FasL-expressing cells (data not shown). Importantly, SHP-1 silencing strongly increased the intensity of cell death using lower dose of JO2 (Figure 1c), and UV-induced cell death was unaffected by the absence of SHP-1 (Figure 1d). Consistent with the increased cell death, higher levels of the cleaved fragments of caspases 8 and 9 and of the poly(ADP)-ribose polymerase (PARP) were detected after Fas triggering in the absence of SHP-1 (Figure 1e). In conclusion, these observations show that SHP-1 has a considerable inhibitory function in Fas-induced cell death in B cells.

SHP-1 constitutive association to Fas negatively regulates the DISC formation. The association of SHP-1 with Fas has been reported for neutrophils.[11] We investigate this association in B cells. By immunoprecipitation, we demonstrate that SHP-1 is constitutively associated with Fas (i.e., before any FasL engagement). Interestingly, this association was reduced after 10 min of activation with FasL, at which point the strong recruitment of FADD and caspase 8 to Fas occurred. It should also be noted that the association of SHP-1 with Fas reappeared again after 30 min of activation (Figure 2). This result suggests the dynamic association of SHP-1 in the DISC formation. Indeed, we found that both FADD and caspase 8 were recruited to the Fas receptor at a significantly higher level in SHP-1 shRNAi cells than in the control shRNAi cells (Figure 2).

Since in the absence of SHP-1, a more intensive DISC formation was observed, we propose that the dissociation of SHP-1 from Fas upon its engagement is required for proper DISC formation.

The tyrosine phosphorylation of Vav is reduced upon Fas activation in an SHP-1-dependent manner. Since SHP-1 is a tyrosine phosphatase, we hypothesized that its inhibitory role in Fas-mediated signaling was connected to its phosphatase activity. We therefore investigated the protein tyrosine phosphorylation pattern following Fas stimulation in the presence or absence of SHP-1. Immunoprecipitation with an anti-p-Tyr antibody (Ab) was carried out after FasL stimulation, and western blotting with an anti-p-Tyr Ab revealed some overphosphorylated proteins in SHP-1-silenced cells with molecular masses of $\sim$150, $\sim$100, $\sim$70, $\sim$52 and $\sim$42 kDa, which were non-phosphorylated or underphosphorylated in the presence of SHP-1 (Figure 3a). These results indicate that, upon Fas stimulation, both kinases and phosphatases play complementary roles, tightly regulating the balance of phosphorylation, and SHP-1 is one of the main actors.[22] In particular, phosphorylation of Vav proteins on several tyrosine residues has been reported.[27] We hypothesized that the 95 kDa guanine nucleotide exchange factor Vav might correspond to the dephosphorylated protein of around 100 kDa, since it has been reported to be an SHP-1 substrate critical in the downregulation of NK cell killing.[17] We therefore analyzed the phosphorylation state of Vav (Figure 3b). Our results demonstrate a high constitutive phosphorylation of Vav, in accordance with data obtained from different B lymphocytes.[22] Moreover, we observed a partial dephosphorylation of Vav following FasL stimulation, suggesting a role of this exchange factor in Fas-induced

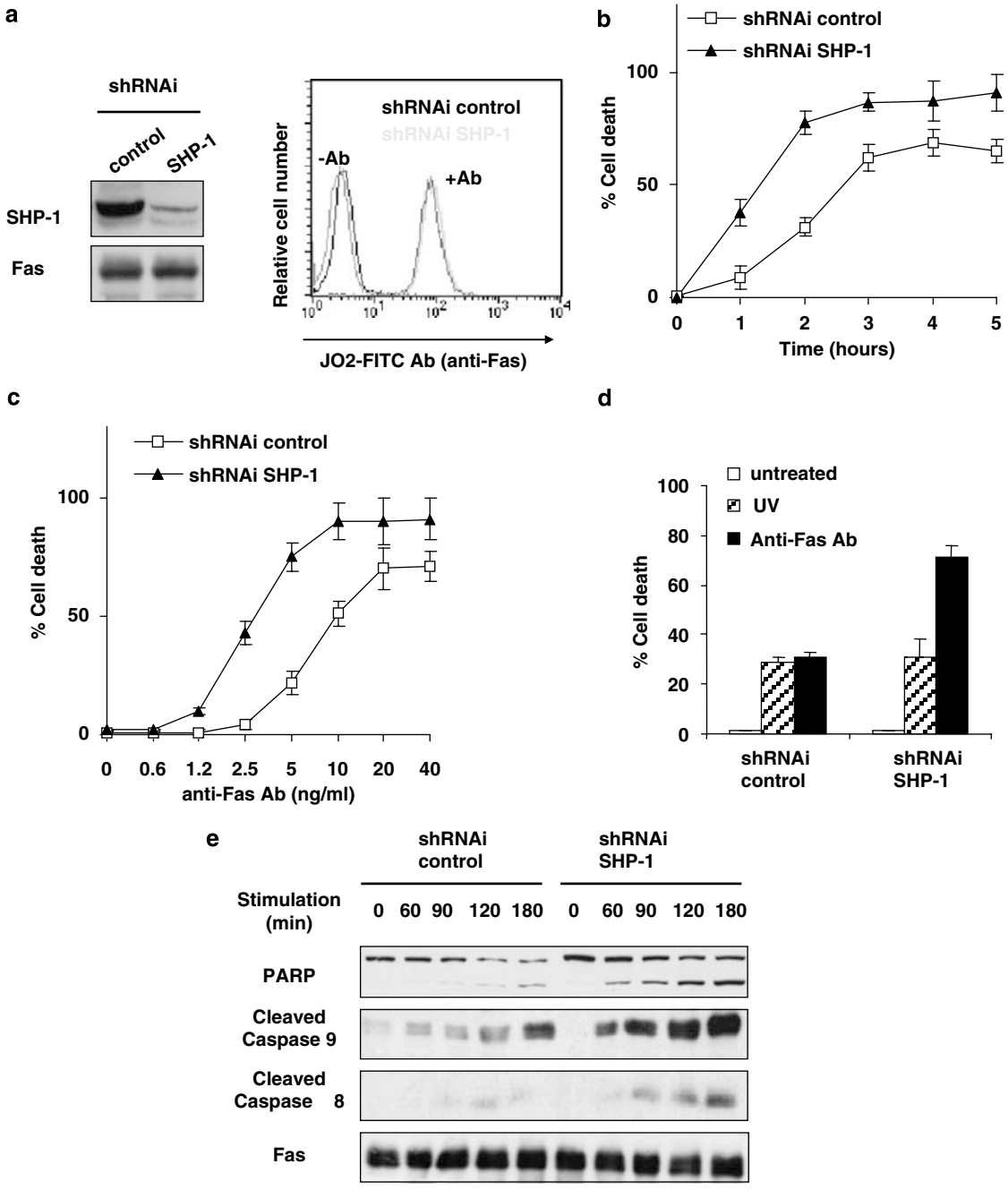

Figure 1 Characterization of the effect of SHP-1 silencing on Fas-induced cell death. (**a**) The shRNAi-induced reduction in SHP-1 was investigated by western blotting of total cell lysates. Flow cytometry analysis was performed with the absence or the presence of FITC-conjugated anti-Fas (JO2) Ab to ensure that cell-surface expression of Fas was equivalent in the control (dark curves) and SHP-1-silenced cells (gray curves). (**b**) Kinetic and (**c**) dose dependence of Fas-induced cell death in control cells (shRNAi control; white square) and SHP-1-silenced cells (shRNAi SHP-1; black triangle). In kinetic experiments, the cells were treated with 100 ng/ml JO2 anti-Fas Ab for the indicated time. The dose dependence was investigated after 3 h of JO2 anti-Fas Ab treatment. (**d**) Comparison of UV-and Fas-mediated cell death in control cells (shRNAi control) and SHP-1-silenced cells (shRNAi SHP-1). DNA fragmentation was observed 24 h after UV treatment (4 mJ, 10 min) (hatched columns), 3 h after Fas Ab treatment (100 ng/ml) (black columns) or on cells left untreated (white columns). (**e**) Cleavage of representative substrates of caspase activation were examined in total cell lysates after 60, 90, 120 and 180 min of activation by FasL, 200 ng/ml Flag-tagged FasL and the anti-Flag Ab M2. A representative western blot from three independent experiments is shown

cell death. To determine whether this dephosphorylation step is an SHP-1-dependent process, we compared the level of tyrosine phosphorylation of Vav in control and SHP-1-silenced cells. While the phosphorylation level was comparable under unstimulated conditions in both cells, Vav remained more strongly phosphorylated upon Fas activation in the SHP-1 shRNAi cells when compared to the control shRNAi cells (Figure 3b). This indicates that, whereas other phosphatases are probably involved in the regulation of the 'steady-state' phosphorylation of Vav, SHP-1 participates in the regulation of the dephosphorylation of Vav following Fas receptor activation.

Vav inhibits Fas-induced cell death in an SHP-1-regulated pathway. Since we found that upon FasL engagement the level of Vav phosphorylation was negatively regulated by SHP-1, we next examined the function of Vav in Fas-induced cell death pathways. We observed that cells transiently transfected with Vav small interference RNA (siRNA) died faster following anti-Fas Ab stimulation compared to control cells transfected with nonspecific oligonucleotides (Figure 4a).

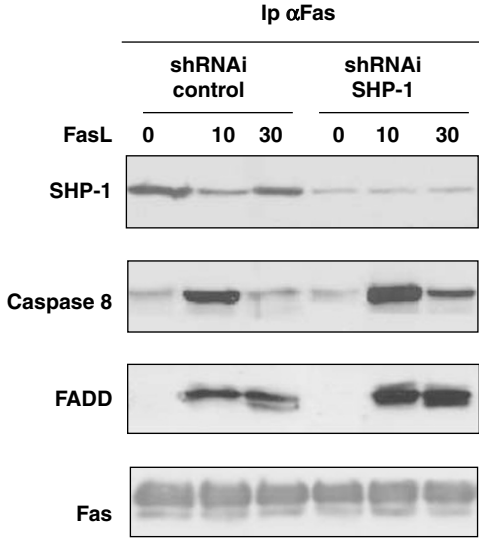

Figure 2 Time dependence of the association of FADD, caspase 8 and SHP-1 with Fas. A total of 5×10^7 cells were activated for 10 or 30 min with 200 ng/ml Flag-tagged FasL and the anti-Flag Ab M2. Following immunoprecipitation from total cell lysates with biotinylated anti-Fas Ab bound to streptavidin–agarose beads, the recruited molecules were detected by western blotting

Further studies revealed that while reducing the amount of Vav induced more extensive cell death upon Fas activation, co-silencing of Vav with SHP-1 had no additive effect (Figure 4a), supporting the hypothesis that SHP-1 and Vav act in the same signaling pathway, downregulating DISC-induced death signaling.

In the presence of SHP-1, overexpression of the Vav protein slightly inhibited Fas-induced cell death (Figure 4b). Indeed, the overexpression of Vav was sufficient to replace the death inhibitory effect of the SHP-1 phosphatase activity and restoring the cell death level of the SHP-1-deficient cells to that of the control cells.

To confirm the crucial role of Vav in Fas-mediated cell death in primary cells, we isolated spleen B cells from control and Vav-knockout mice and compared their sensitivity to Fas-induced cell death upon FasL stimulation. As resting B cells express very low levels of Fas, we preinjected the mice with FITC–BSA 1 week before B lymphocyte isolation. Importantly, whereas no difference in the level of Fas expression at the cell surface of control and Vav-knockout B cells could be detected (data not shown), Fas-induced cell death was significantly increased in the absence of Vav, in accordance with the results obtained with the silenced cell line (Figure 4c).

SHP-1 influences Fas-induced cell death by modulating Fas–actin cytoskeleton connection. Since Vav proteins are involved in regulating cytoskeletal dynamics in response to extracellular stimuli[20,28] and an intact actin cytoskeleton is required for DISC formation,[9,25] we hypothesized that the regulatory effect of SHP-1 could be executed through the regulation of cytoskeletal rearrangements. We therefore compared the kinetics of Fas-induced cell death in the presence of latrunculin A (LtnA), an inhibitor of actin polymerization reported to abrogate Fas-mediated cell

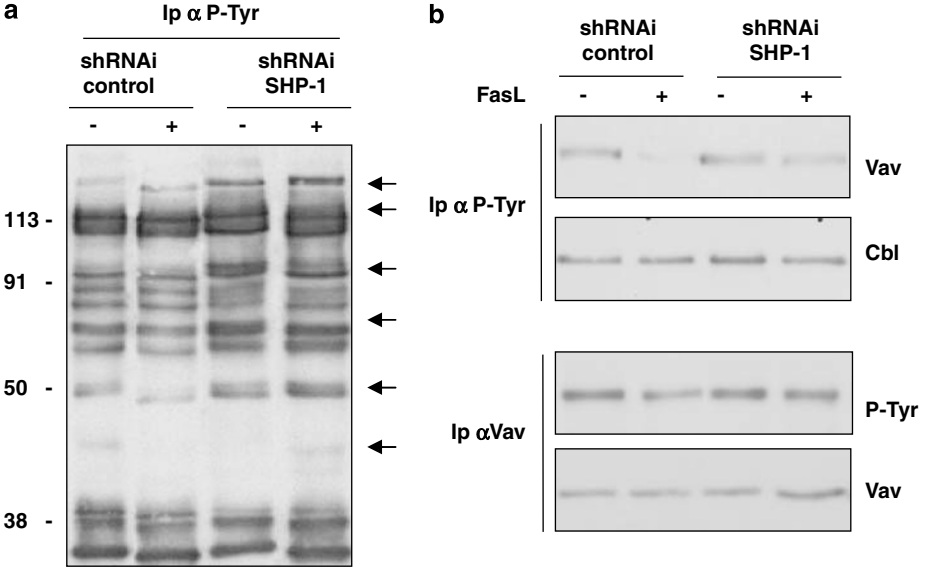

Figure 3 The absence of SHP-1 induces overphosphorylation upon Fas stimulation. (**a**) A total of 2×10^7 cells were activated with 200 ng/ml Flag-tagged FasL and the anti-Flag Ab M2 for 15 min. Immunoprecipitates from total cell lysates with anti-p-Tyr were separated by SDS-PAGE and subjected to western blotting for phosphotyrosine. (**b**) After 15 min activation, the intensity of Vav tyrosine phosphorylation was measured by immunoprecipitation with anti-p-Tyr or anti-Vav Abs bound to protein A/G. The immunoprecipitates were immunoblotted with the indicated Abs. The figures show representative experiments from at least three independent experiments

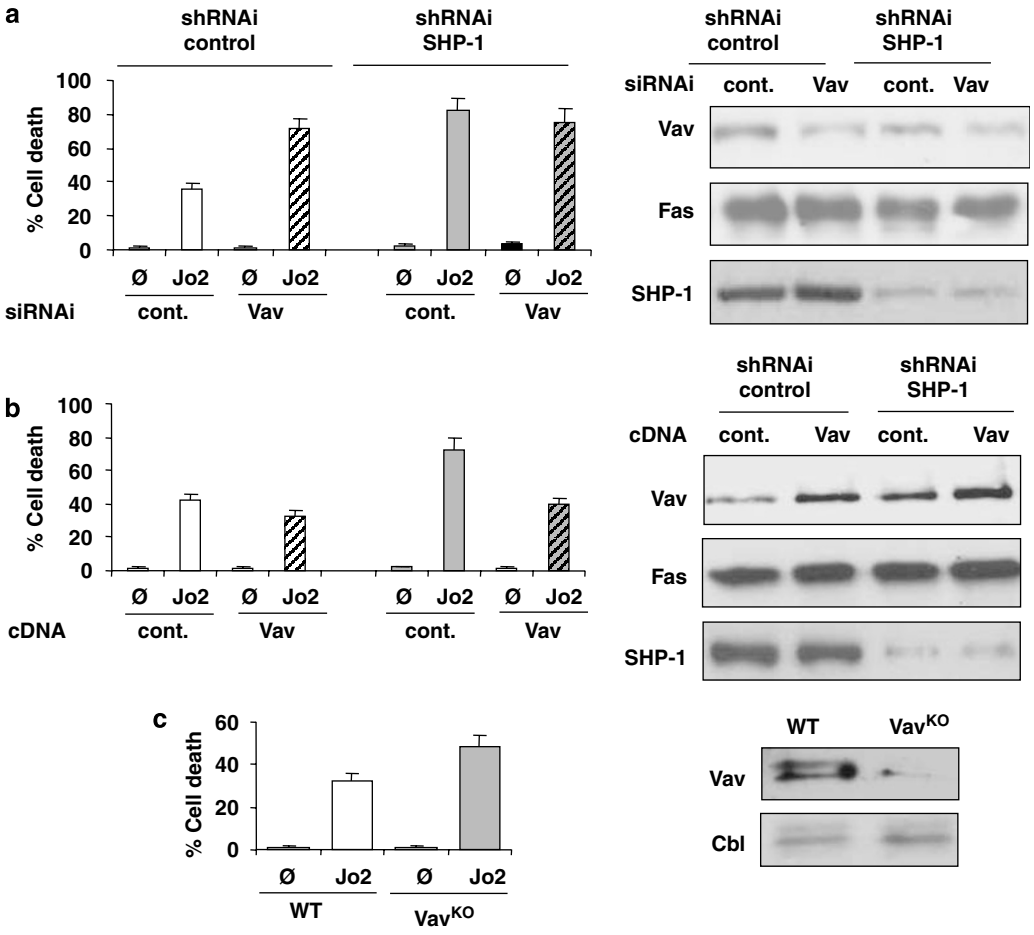

Figure 4 Vav is required for Fas-induced cell death in an SHP-1-dependent manner. Cell clones expressing or lacking SHP-1 were subjected to transient transfection with Vav-specific RNAi (**a**) or with Vav cDNA (**b**). The amount of Vav was compared by western blotting of total cell lysates. Cells were treated with 100 ng/ml anti-Fas Ab for 2 h and Fas-induced cell death was measured by PI staining. (**c**) Fas-induced cell death in B cells separated from control Black/6 mice and Vav-knockout mice was compared. Cell death of the purified B cells (at least 95% of the cells were B220 positive) was induced with 40 ng/ml anti-Fas Ab for 24 h and measured by PI staining

death,[9] in the absence (SHP-1 shRNAi) or presence of SHP-1 (control shRNAi). We observed that to obtain a comparable cell death inhibition in both cell lines, a five-fold greater concentration of LtnA is required when SHP-1 was absent from the cells (Figure 5a), suggesting that SHP-1 is an important negative regulator of the cytoskeletal rearrangements occurring during Fas-mediated signaling.

We next quantitatively examine Fas internalization, a process tightly regulated by cytoskeletal rearrangements, lying upstream of the DISC formation and indispensable for its proper formation.[8,9] We synchronized Fas activation by incubating SHP-1 shRNAi and control shRNAi cells at 4°C with rhFasL-Flag cross-linked with mouse anti-Flag Ab before raising the temperature (37°C). The amount of Fas remaining on the cell surface, representing the uninternalized Fas, was then quantified. We observed an increase in Fas internalization in the absence of SHP-1, indicating that SHP-1 is involved in negatively regulating Fas receptor internalization upon engagement by its ligand (Figure 5b).

To confirm that the downregulatory effect of SHP-1 is executed through the control of cytoskeleton rearrangements, the ability of Fas to bind to actin in control and SHP-1-silenced cells was examined. In control cells, a binding of actin to Fas was detected upon 10 min of FasL stimulation, whereas this association completely disappears after 30 min of activation (Figure 5c). In contrast, both a more intensive (at 10 min) and prolonged (at 30 min) actin/Fas connection was detected in SHP-1-silenced cells (Figure 5c), leading to the conclusion that the regulation of the Fas–actin complex formation was dependent on SHP-1.

Taken together, these data suggest that upon FasL engagement, the Fas–SHP-1 dissociation regulates the DISC formation, and therefore the Fas-mediated cell death through the regulation of Fas–actin connection.

Our results identifying Vav as a crucial actor in the SHP-1-mediated Fas signaling (Figures 3 and 4), together with both the well-known role of Vav in cytoskeletal rearrangement, and its ability to interact with actin,[29] led us to investigate the potential modulatory function of the Vav–actin connection upon Fas engagement. Immunoprecipitation by anti-actin Ab revealed a constitutive Vav–actin association that nearly completely disappeared at 10 min after FasL stimulation, before becoming detectable again later (30 min) (Figure 5d). It must be noted that the kinetics of Vav–actin association,

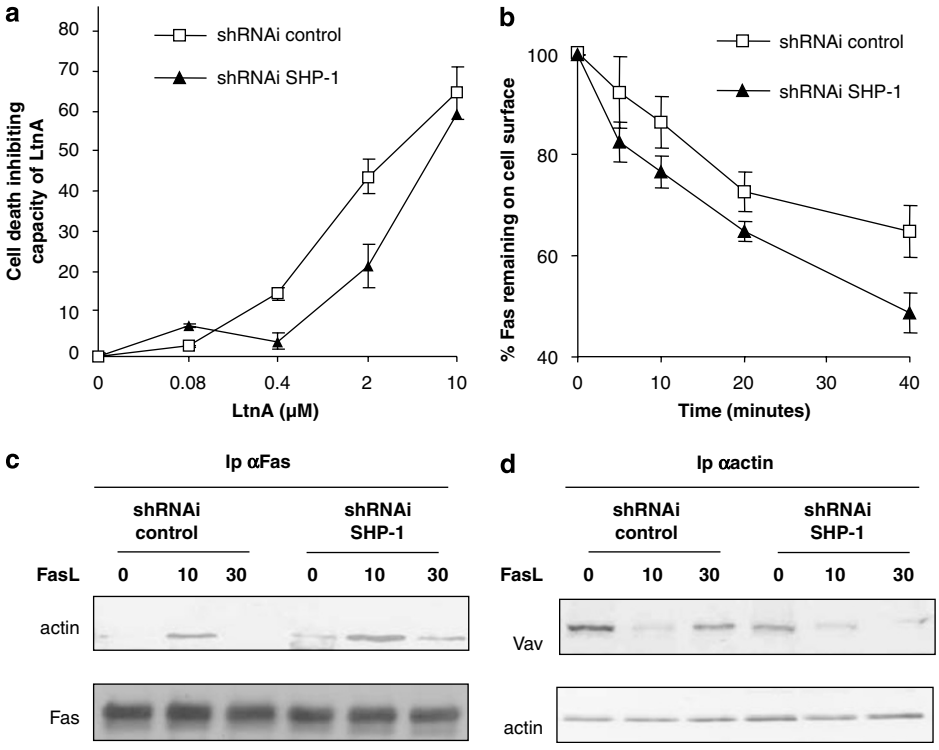

Figure 5 SHP-1 inhibits the internalization of the Fas receptor through the regulation of actin-dependent signaling. (**a**) After 30 min pretreatment with different concentrations of LtnA, the cells were activated with 100 ng/ml anti-Fas Ab for 3 h and cell death was measured by flow cytometry. The percentage of cell death obtained without pretreatment with LtnA, both in the presence and absence of SHP-1, was defined as maximal cell death (max cell death), whereas the one obtained following LtnA pretreatment was referred as cell death LtnA. The inhibitory capacity of LtnA was calculated as cell death LtnA $\times$ 100/max cell death. Data represent the average of least three independent experiments. (**b**) Cells stably expressing SHP-1 shRNAi or control shRNAi were subjected to synchronized activation and internalization assessment. Data represent the average of least three independent experiments. (**c**) The Fas–actin and (**d**) Vav–actin association were studied in control cells (control shRNAi) and SHP-1-silenced cells (SHP-1 shRNAi) by immunoprecipitation with biotinylated anti-Fas or anti-actin Abs bound to streptavidin–agarose or A/G sepharose beads, respectively, followed by detection of molecules by western blotting

before and after Fas engagement, represents the mirror image of Fas–actin association (Figure 5c).

Importantly, this reassociation of Vav to actin is not detectable in SHP-1-silenced cells (Figure 5d), explaining the sustained formation of Fas–actin association after prolonged stimulation (Figure 5c).

All together, these findings suggest that following Fas stimulation, Vav, through the regulation by the phosphatase activity of SHP-1, competes with Fas in the binding to actin, thereby attenuating the cell death signal. In the absence of SHP-1, and upon long-term Fas stimulation, since Vav cannot compete with Fas for its interaction with actin, an exaggerated Fas internalization and DISC formation lead to a more extensive cell death.

Ezrin competes with Vav for actin binding. Since the kinetic comparison of Vav and Fas association with actin strongly supports the hypothesis that Vav competes with Fas for actin binding, we investigated in this context the role of ezrin, a well-known linker between Fas and the actin cytoskeleton, recently reported critical for Fas internalization, DISC formation and cell death.[9]

We first compared the ezrin–Fas interaction in the presence or in the absence of SHP-1. In control cells, the connection was barely detectable upon 10 min activation and weakly detectable after 30 min (Figure 6a). In total contrast, in SHP-1-silenced cells, a strong binding between Fas and ezrin was detectable as early as 10 min after activation, whereas a consequent decrease of association was already visible 30 min after activation (Figure 6a). To confirm the possibility that Vav and ezrin compete for actin binding, after 30 min of Fas activation, we compared the amount of actin co-immunoprecipitated by anti-Vav Ab in control cells or in cells overexpressing ezrin. Nearly no actin could be found associated to Vav in cells overexpressing ezrin, demonstrating that an increasing amount of ezrin is able to compete with Vav for its actin binding (Figure 6b).

Based on the observation that the association of ezrin with Fas was increased in the absence of SHP-1 (Figure 6a), we hypothesized that overexpression of ezrin may have a similar functional effect as SHP-1 silencing. We therefore investigated Fas-induced cell death in cells transiently transfected with ezrin cDNA. We found that this is indeed the case since ezrin overexpression led to more extensive cell death in shRNAi control cells (Figure 6c). Interestingly, this increase was not detectable in SHP-1 shRNAi cells, where a decreased Vav–actin connection was responsible of cell death enhancement. The lack of additive effect between both situations (ezrin overexpression and SHP-1 silencing) reinforces the idea that they both act at the same functional level.

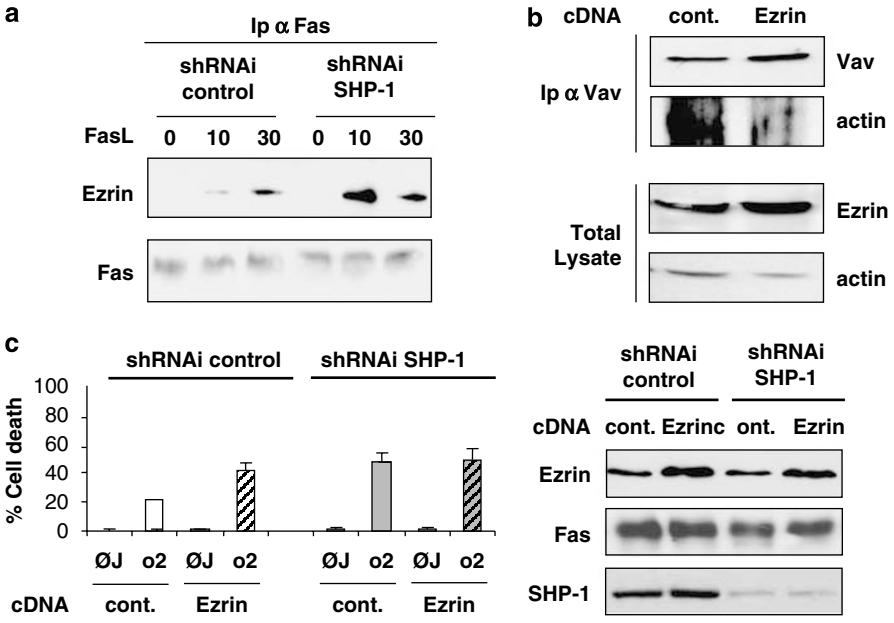

Figure 6 Ezrin compete with Vav for binding to actin upon FasL stimulation. (**a**) The Fas–ezrin association was determined by immunoprecipitation with biotinylated anti-Fas Ab bound to streptavidin–agarose beads. The immunoprecipitates were immunoblotted with anti-ezrin Ab. (**b**) The Vav–ezrin competition was investigated in transiently transfected A20 cells with ezrin cDNA. Twenty-four hours after transfection, the cells were activated for 30 min with 200 ng/ml FasL, and the Vav–actin association was determined by immunoprecipitation with anti-Vav Ab bound to protein A/G beads. The immunoprecipitates were immunoblotted with the indicated Abs. Immunoblots on total cell lysates indicate the total amount of ezrin. (**c**) Cells expressing or lacking SHP-1 were subjected to transient transfection with ezrin cDNA. The amount of ezrin was compared by western blotting of total cell lysates. Cell death was induced by treating cells with 100 ng/ml anti-Fas Ab for 2 h and measured by PI staining

All together, our data provide evidence that the SHP-1-mediated pathway negatively regulates the connection between Fas and the cytoskeleton through Fas–actin interaction therefore modulating receptor internalization and subsequently DISC formation and cell death.

Discussion

Expression of a chimeric protein comprised of the Fas extracellular domain and the FADD DED in lymphocytes of Fas-deficient MRL-*lpr/lpr* mice completely diminishes their T cell but not their B-cell abnormalities, suggesting that, whereas FADD might be sufficient for initiation of Fas signaling in primary T cells, other essential pathways might operate in B lymphocytes.[30] Although several Fas-associated proteins have been identified, we specifically focused our interest on one of them, the SHP-1 tyrosine phosphatase, for which no functional involvement in Fas-mediated signaling was reported. Our results strongly indicate that silencing of SHP-1 can enhance Fas-mediated cell death through an enhancement of DISC formation. More importantly, the increased association of FADD and caspase 8 with Fas in the absence of this phosphatase indicates that the action of SHP-1 lies upstream of DISC formation. Inhibition of Fas-mediated cell death was originally reported for lymphoid cells derived from the SHP-1-negative motheaten mice.[31] Nevertheless, this study did not examine Fas-mediated cell death in B cells upon antigen activation, a condition necessary for their sensitivity to this pathway, as it allows a significant increase in Fas expression on the cell surface. The involvement of SHP-1 in Fas-mediated cell death is also controversial in T cells: whereas Su *et al*. reported a positive role of SHP-1, others were not able to detect any difference.[32] Our results suggest that, at least in B cells, SHP-1 has a critical negative role in Fas-mediated cell death.

The great importance of the actin cytoskeleton in the clathrin-dependent internalization of the receptor is now clearly established.[7–9] Having identified both a constitutive pre-ligand Fas/SHP-1 interaction and an inhibitory function of SHP-1 at the level or upstream of DISC formation, we hypothesized that this protein might play a role in the regulation of the actin cytoskeletal rearrangements occurring after FasL engagement. We show a crucial negative role of SHP-1 in the regulation of the Fas-mediated cytoskeletal rearrangement, since the internalization of Fas was accelerated when the phosphatase was absent and that the inhibitory effect of LntA on cell death was significantly reduced, in SHP-1 shRNAi cells. We compared the Fas–ezrin association in control shRNAi and SHP-1 shRNAi cells and observed that the association was greater in the absence of SHP-1, indicating that the mechanisms by which SHP-1 regulates the DISC formation depend of actin cytoskeletal rearrangements.

Vav is a well-known substrate of SHP-1, which regulates cell death signaling.[17] We showed that Vav phosphorylation is negatively regulated upon FasL engagement and that this process partially depends on the presence of SHP-1. It is well documented that functions of Vav are tightly regulated by its tyrosine phosphorylation.[21] The dephosphorylation of Vav suggesting a regulatory role of this Rho GTPase in Fas-mediated cell death, we investigated the effect of Vav silencing on Fas-induced signaling. We show that Fas-induced cell death increased with the decrease in the

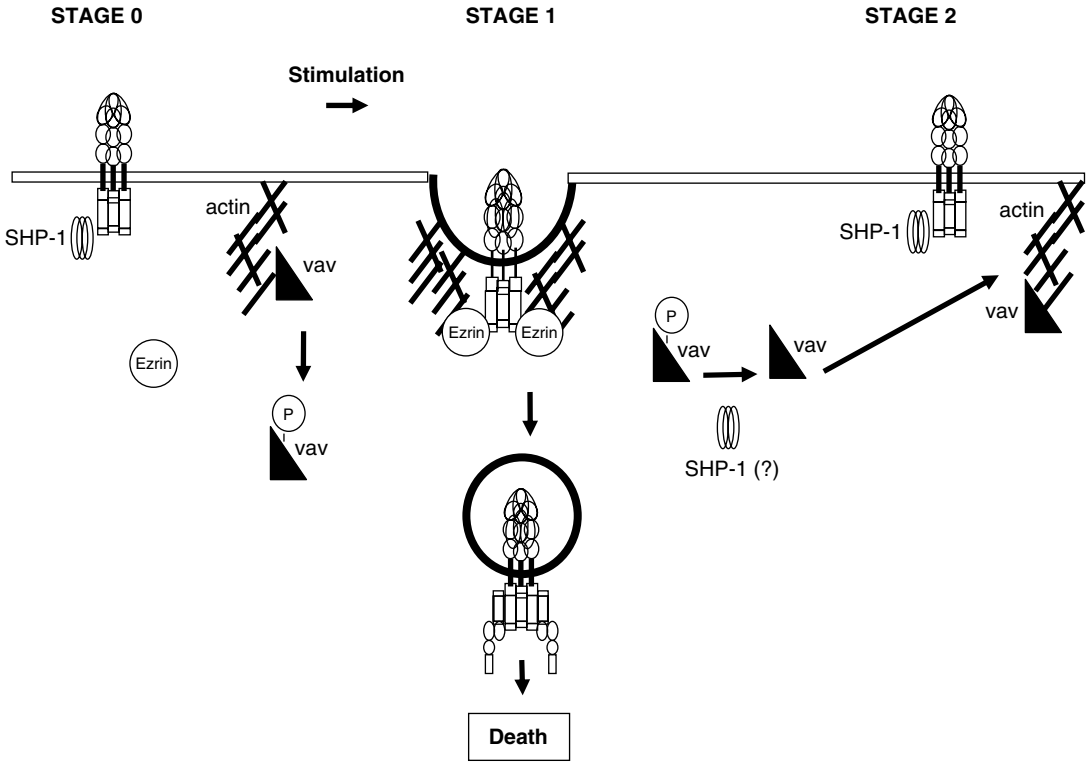

Figure 7 Fine-tuning downregulation of Fas mediated cell death by SHP-1-controlled cytoskeleton rearrangement. Based on our results, we suggest a model in which SHP-1 is responsible for the downregulation of the Fas-mediated cell death. In non-stimulated cells, SHP-1 is constitutively associated with Fas, and Vav is constitutively connected to actin. Upon FasL stimulation, both SHP-1 and Vav are dissociated from their partner molecules allowing the binding of ezrin to actin, and subsequently the receptor internalization, DISC formation and cell death. The reassociation of SHP-1 leads to dephosphorylation of Vav. This allows Vav to compete with Fas in the binding with the actin and thereby terminating the receptor internalization and the DISC formation. In the absence of SHP-1, the lack of Vav dephosphorylation allows a prolonged Fas–actin connection, leading to more intensive DISC formation and enhanced cell death

level of Vav, demonstrating an inhibitory role for the Vav protein in both B cell line and primary B cells. As the Vav silencing had no effect in the absence of SHP-1, the two molecules must participate in the same signaling pathway. Our results suggest the regulation of the Vav dephosphorylation by SHP-1 as a restraining factor of Fas-induced cell death, and that this negative regulation by the phosphatase activity of SHP-1 must be lifted to allow Vav phosphorylation to obtain a full activation of Fas-mediated cell death pathway.

By analyzing the various interactions of all the investigated molecules during the course of Fas signaling, we observed different stages (Figure 7):

(i) *Stage 0*: Two constitutive associations (i.e., even prior to any receptor engagement): one between Fas and SHP-1, another between Vav and actin.
(ii) *Stage 1*: Upon FasL activation, in an early time window, these two interactions are rapidly reduced, whereas Fas binds to actin, ezrin, FADD and caspase 8: this is the time window where both internalization and then DISC formation occur allowing the cell death signaling propagation.
(iii) *Stage 2*: In a later time window, a 'back to non-stimulated conditions (stage 0)' is found: the Fas–actin–ezrin and Fas–FADD–caspase 8 complexes are dissociated, whereas the Vav–actin and Fas–SHP-1 interactions reappear.

In the absence of SHP-1, the molecular interactions associated to stage 2 are missing and the interactions found at this step of activation are qualitatively equivalent to the ones found in stage 1 and quantitatively exacerbated, explaining a more pronounced Fas internalization and cell death.

Our data provide evidence that Fas-induced cell death and DISC formation is tightly regulated by SHP-1. Upon Fas engagement, the SHP-1-driven Vav dephosphorylation, by downmodulating the Fas–ezrin–actin linkage, is a fine-tune switch-off mechanism that the cell uses as a way to terminate the receptor internalization, controlling therefore the time and extent of the DISC formation and cell death (Figure 7).

Materials and Methods

Antibodies and reagents. The Abs used were anti-PARP (BioMol), anti-caspase 9 (MBL), anti-FLAG M2, anti-Fas M20, anti-Vav (Santa Cruz), anti-actin (Sigma), anti-p-Tyr 4G10 (Upstate), anti-FADD (Calbiochem), anti-SHP-1 (Becton Dickinson/Transduction Laboratories), anti-Fas (clone JO2) (Becton Dickinson/Transduction Laboratories), JO2–FITC (BD Bioscience). The anti-caspase 8 was a kind gift from Dr. Hakem, B220 and anti-CD3 Ab were a kind gift from Dr. Sarmay, and anti-Thy-1.2 Ab and rabbit complement were a kind gift from Dr. Prechl. The horseradish peroxidase-conjugated anti-rabbit or anti-mouse Abs were from Jackson ImmunoResearch. Recombinant human FasL and streptavidin–agarose beads from Sigma-Aldrich, protein A/G Plus-agarose beads from Santa Cruz and

LtnA from Sigma; peroxidase-labeled Abs were detected using the Pierce ECL reagent.

siRNA and construction of expression vectors. The gene-specific insert, containing sequences corresponding to nucleotides 957–976 (CAAGAACCGCTACAAGAAC) of mouse SHP-1 and its reverse complement, which were separated from each other by a nine-nucleotide non-complementary spacer (ttcaagaga), was cloned into the mammalian expression vector pSuper (OligoEngine). This vector is referred to as pSuper shRNAi SHP-1. The control shRNAi vector was previously described.[9] The Vav was silenced by RNAi nucleotides corresponding to nucleotides 1563–1582 (GAAGU GGAUGGAACAGUUC) of mouse Vav1. The cDNA encoding Myc-tagged Vav1 and VSV-tagged ezrin were previously described.[33,34]

A20.2J cell transfection. A20.2J (A20) mouse B cells expressing short hairpin (sh) SHP-1 siRNA or control siRNA were established by transfecting cells at 300 V for 20 ms with 20 μg of mouse SHP-1 shRNAi (shRNAi SHP-1) or control shRNAi (shRNAi control) using the Amaxa Nucleofection Technology™ (Amaxa, Cologne, Germany) and stable cells were selected with geneticin. For transient transfection, cells were suspended in 0.5 ml complete RPMI medium and transfected with 100 nM Vav (GAAGUGGAUGGAACAGUUC) RNAi sequences at 260 V for 20 ms. The cells were resuspended in fresh media and the transfection was repeated after 24 h incubation. After another 24 h, the protein expression was determined by western blotting and the apoptosis assay was carried out.

Mouse B lymphocyte isolation and purification. The Vav1-deficient mice[35] or control Black/6 mice were preinjected with 100 μl of 1 mg/ml FITC–BSA plus 100 μl complete Freund's adjuvant 1 week before B-cell isolation, as previously described.[36] Briefly, the spleen was removed and, after lysis of the red blood cells, the remaining cells were washed and treated with anti-Thy-1.2 Abs for 30 min at room temperature and washed once more. The cells were further incubated with RPMI 1640 medium containing rabbit complement for 30 min at 37°C. The percentage of B cells was analyzed by flow cytometry using fluorescently labeled anti-B220 and anti-CD3 Abs.

Flow cytometry. A20 cells (10^6 cells/100 μl) were surface-stained with FITC-labeled anti-Fas (JO2) mAb (BD Bioscience) for 1 h on ice. Cells were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson) using CellQuest software (Becton Dickinson).

Internalization assays. Assessment was performed after synchronized stimulation. SHP-1 shRNAi and control shRNAi A20 cells were incubated with 200 ng/ml rhFasL with 1 μg/ml mouse anti-Flag IgG Ab (M2) or with M2 only (control for background binding) on ice for 30 min.

After two washes with ice-cold medium to remove unbound FasL, cells were transferred at 37°C for the indicated time to trigger synchronized Fas stimulation or were kept on ice (control, 0 min activation). The activation was stopped by adding ice-cold medium. Cells were then washed and incubated 30 min on ice with FITC-conjugated anti-JO2. To determine the degree of internalization of Fas–FasL complexes, which corresponded to the decrease in surface-bound anti-Fas(JO2) Ab, the mean fluorescence intensity (MFI) of the background control cells (incubated with M2 only) was subtracted from the MFI of the FasL-bound cells at each time point to obtain the $MFI_{absolute}$. The $MFI_{absolute}$ of stimulated cells was divided by the $MFI_{absolute}$ of control non-activated cells ($t = 0$) to obtain the percentage of Fas remaining on the cell surface.

Cell death experiments. The sensitivity of A20 cells (10^6 cells/ml) to Fas-mediated cell death was examined after incubating the cells with 100 ng/ml of JO2 anti-Fas Ab at 37°C. After the indicated time, the apoptotic rate in the cell suspension was detected by flow cytometric analysis. Briefly, cells were fixed in ice-cold 70% ethanol, washed in 38 mM sodium citrate (pH 7.4) and stained for 20 min at 37°C with 69 μM propidium iodide (Sigma) and 5 μg/ml RNase A (Sigma) in 38 mM sodium citrate (pH 7.4). Cells were analyzed with a flow cytometer (FACSCalibur; Becton Dickinson), and the proportion of apoptotic cells represented by the subG1 peak was determined.[4] For LtnA treatment, cells were pretreated for 30 min in RPMI medium containing the indicated amount of LtnA (Calbiochem).

Immunoprecipitation and western blotting. Immunoprecipitation experiments were performed on A20 cells (10^8) stimulated with 250 ng/ml Flag-rhFasL plus 2 μg/ml M2 Ab for 10 or 30 min at 37°C, or left unstimulated. Cells were resuspended in lysis buffer (25 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml chymostatin and 5 μg/ml α-2 macroglobulin) and sonicated (5 × 5 s). The resulting postnuclear supernatant was then subjected to immunoprecipitation at 4°C overnight with biotinylated JO2 anti-Fas Ab coupled to streptavidin–agarose beads (Sigma), or with anti-p-Tyr Ab or anti-SHP-1 Ab coupled to protein A/G agarose beads (Santa Cruz). The beads were washed four times with lysis buffer, and the immunoprecipitates were eluted from beads by heating in Laemmli buffer at 95°C for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with the indicated Abs. Western blot analysis of proteins was performed according to standard protocols. Briefly, cells were resuspended in Laemmli buffer. After sonication and denaturing (95°C, 5 min), the solubilized proteins were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Molsheim, France) by electroblotting.

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1. Krammer PH. CD95's deadly mission in the immune system. *Nature* 2000; **407**: 789–795.
2. Peter ME, Krammer PH. The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 2003; **10**: 26–35.
3. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH *et al.* Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 1995; **14**: 5579–5588.
4. Hueber AO, Bernard AM, Herincs Z, Couzinet A, He HT. An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. *EMBO Rep* 2002; **3**: 190–196.
5. Henkler F, Behrle E, Dennehy KM, Wicovsky A, Peters N, Warnke C *et al.* The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular FasL–Fas clusters of high stability. *J Cell Biol* 2005; **168**: 1087–1098.
6. Miyaji M, Jin ZX, Yamaoka S, Amakawa R, Fukuhara S, Sato SB *et al.* Role of membrane sphingomyelin and ceramide in platform formation for Fas-mediated apoptosis. *J Exp Med* 2005; **202**: 249–259.
7. Algeciras-Schimnich A, Shen L, Barnhart BC, Murmann AE, Burkhardt JK, Peter ME. Molecular ordering of the initial signaling events of CD95. *Mol Cell Biol* 2002; **22**: 207–220.
8. Lee KH, Feig C, Tchikov V, Schickel R, Hallas C, Schutze S *et al.* The role of receptor internalization in CD95 signaling. *EMBO J* 2006; **25**: 1009–1023.
9. Chakrabandhu K, Herincs Z, Huault S, Dost B, Peng L, Conchonaud F *et al.* Palmitoylation is required for efficient Fas cell death signaling. *EMBO J* 2007; **26**: 209–220.
10. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 1992; **356**: 314–317.
11. Daigle I, Yousefi S, Colonna M, Green DR, Simon HU. Death receptors bind SHP-1 and block cytokine-induced anti-apoptotic signaling in neutrophils. *Nat Med* 2002; **8**: 61–67.
12. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* 1999; **17**: 875–904.
13. Pani G, Kozlowski M, Cambier JC, Mills GB, Siminovitch KA. Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling. *J Exp Med* 1995; **181**: 2077–2084.
14. Singer BB, Klaile E, Scheffrahn I, Muller MM, Kammerer R, Reutter W *et al.* CEACAM1 (CD66a) mediates delay of spontaneous and Fas ligand-induced apoptosis in granulocytes. *Eur J Immunol* 2005; **35**: 1949–1959.
15. Mizuno K, Tagawa Y, Mitomo K, Watanabe N, Katagiri T, Ogimoto M *et al.* Src homology region 2 domain-containing phosphatase 1 positively regulates B cell receptor-induced apoptosis by modulating association of B cell linker protein with Nck and activation of c-Jun NH2-terminal kinase. *J Immunol* 2002; **169**: 778–786.
16. Tsui FW, Martin A, Wang J, Tsui HW. Investigations into the regulation and function of the SH2 domain-containing protein-tyrosine phosphatase, SHP-1. *Immunol Res* 2006; **35**: 127–136.
17. Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. *Mol Cell Biol* 2003; **23**: 6291–6299.

18. Baba T, Fusaki N, Shinya N, Iwamatsu A, Hozumi N. Actin tyrosine dephosphorylation by the Src homology 1-containing protein tyrosine phosphatase is essential for actin depolymerization after membrane IgM cross-linking. *J Immunol* 2003; **170**: 3762–3768.
19. Baba T, Fusaki N, Shinya N, Iwamatsu A, Hozumi N. Myosin is an *in vivo* substrate of the protein tyrosine phosphatase (SHP-1) after mIgM cross-linking. *Biochem Biophys Res Commun* 2003; **304**: 67–72.
20. Hornstein I, Alcover A, Katzav S. Vav proteins, masters of the world of cytoskeleton organization. *Cell Signal* 2004; **16**: 1–11.
21. Bustelo XR. Vav proteins, adaptors and cell signaling. *Oncogene* 2001; **20**: 6372–6381.
22. Madureira PA, Matos P, Soeiro I, Dixon LK, Simas JP, Lam EW. Murine gammaherpesvirus 68 latency protein M2 binds to Vav signaling proteins and inhibits B-cell receptor-induced cell cycle arrest and apoptosis in WEHI-231 B cells. *J Biol Chem* 2005; **280**: 37310–37318.
23. Tuosto L, Marinari B, Piccolella E. CD4-Lck through TCR and in the absence of Vav exchange factor induces Bax increase and mitochondrial damage. *J Immunol* 2002; **168**: 6106–6112.
24. Mimura T, Minota S, Nojima Y, Morino N, Hamasaki K, Furuya H *et al.* Constitutive tyrosine phosphorylation of the vav proto-oncogene product in MRL/Mp-lpr/lpr mice. *J Immunol* 1997; **158**: 2977–2983.
25. Algeciras-Schimnich A, Peter ME. Actin dependent CD95 internalization is specific for Type I cells. *FEBS Lett* 2003; **546**: 185–188.
26. Parlato S, Giammarioli AM, Logozzi M, Lozupone F, Matarrese P, Luciani F *et al.* CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway. *EMBO J* 2000; **19**: 5123–5134.
27. Tybulewicz VL. Vav-family proteins in T-cell signalling. *Curr Opin Immunol* 2005; **17**: 267–274.
28. Fischer KD, Tedford K, Penninger JM. Vav links antigen-receptor signaling to the actin cytoskeleton. *Semin Immunol* 1998; **10**: 317–327.
29. Castresana J, Saraste M. Does Vav bind to F-actin through a CH domain? *FEBS Lett* 1995; **374**: 149–151.
30. Kabra NH, Cado D, Winoto A. A tailless fas-FADD death-effector domain chimera is sufficient to execute Fas function in T cells but not B cells of MRL-lpr/lpr mice. *J Immunol* 1999; **162**: 2766–2774.
31. Su X, Zhou T, Wang Z, Yang P, Jope RS, Mountz JD. Defective expression of hematopoietic cell protein tyrosine phosphatase (HCP) in lymphoid cells blocks Fas-mediated apoptosis. *Immunity* 1995; **2**: 353–362.
32. Takayama H, Lee MH, Shirota-Someya Y. Lack of requirement for SHP-1 in both Fas-mediated and perforin-mediated cell death induced by CTL. *J Immunol* 1996; **157**: 3943–3948.
33. Crepaldi T, Gautreau A, Comoglio PM, Louvard D, Arpin M. Ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells. *J Cell Biol* 1997; **138**: 423–434.
34. Tartare-Deckert S, Monthouel MN, Charvet C, Foucault I, Van Obberghen E, Bernard A *et al.* Vav2 activates c-fos serum response element and CD69 expression but negatively regulates nuclear factor of activated T cells and interleukin-2 gene activation in T lymphocyte. *J Biol Chem* 2001; **276**: 20849–20857.
35. Turner M, Mee PJ, Walters AE, Quinn ME, Mellor AL, Zamoyska R *et al.* A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity* 1997; **7**: 451–460.
36. Norvell A, Monroe JG. Acquisition of surface IgD fails to protect from tolerance-induction. Both surface IgM- and surface IgD-mediated signals induce apoptosis of immature murine B lymphocytes. *J Immunol* 1996; **156**: 1328–1332.