Vav family proteins constitute disparate branching points for distinct BCR signaling pathways

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Antigen recognition by B-cell antigen receptors (BCRs) activates distinct intracellular signaling pathways that control the differentiation fate of activated B lymphocytes. BCR-proximal signaling enzymes comprise protein tyrosine kinases, phosphatases, and plasma membrane lipid-modifying enzymes, whose function is furthermore coordinated by catalytically inert adaptor proteins. Here, we show that an additional class of enzymatic activity provided by guanine-nucleotide exchange factors (GEFs) of the Vav family controls BCR-proximal Ca\(^{2+}\) mobilization, cytoskeletal actin reorganization, and activation of the PI3 kinase/Akt pathway. Whereas Vav1 and Vav3 supported all of those signaling processes to different extents in a human B-cell model system, Vav2 facilitated actin remodeling, and activation of Akt but did not promote Ca\(^{2+}\) signaling. On BCR activation, Vav1 was directly recruited to the phosphorylated BCR and to the central adaptor protein SLP65 via its Src homology 2 domain. Pharmacological inhibition or genetic inactivation of the substrates of Vav GEFs, small G proteins of the Rho/Rac family, impaired BCR-induced Ca\(^{2+}\) mobilization, probably because phospholipase C\(\gamma\) 2 requires activated Rac proteins for optimal activity. Our findings show that Vav family members are key relays of the BCR signalosome that differentially control distinct signaling pathways both in a catalysis-dependent and -independent manner.

Keywords: antigen receptors · B cells · BCR · Ca\(^{2+}\) signaling · Vav family proteins

Additional supporting information may be found online in the Supporting Information section at the end of the article.
position 204 (Y204) of Igα [5, 6]. Tyrosine-phosphorylated SLP65 serves as a docking platform for both Bruton’s tyrosine kinase (Btk) and its immediate substrate phospholipase Cγ2 (PLCγ2) [7, 8]. This process of PLCγ2 activation can furthermore be supported by accessory proteins such as Grb2 and CIN85 [9, 10].

Activated PLCγ2 hydrolyzes the plasma membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2), thus, generating the second messenger molecules diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). Plasma membrane-bound DAG recruits C1 domain-containing signaling proteins like protein kinase C and Ras guanine nucleotide release proteins, whereas the soluble IP3 induces opening of IP3-gated Ca2+ channels in the membrane of the endoplasmic reticulum called IP3 receptors (IP3R), allowing Ca2+ ions to enter the cytosol [5]. This first wave of Ca2+ influx into the cytosol is linked to a second wave controlled by store-operated Ca2+ channels in the plasma membrane [11]. Both the intensity and duration of BCR-induced Ca2+ mobilization are tuned by a variety of activating or inhibiting cell surface coreceptors and by intracellular enzymes and adaptor proteins [12, 13]. The mobilization of the second messenger Ca2+ and the activation of BCR signaling in general were furthermore reported to be under control of the cortical actin cytoskeleton as treatment of certain B cells with actin filament destabilizing compounds can cause a “BCR-like” Ca2+ signal [14, 15]. However, this situation may be restricted to B cells that coexpress mlgD-BCRs, CD19, and the chemokine receptor CXCR4 together on their cell surface [16]. Whatever the case may be, the integrated activity of cellular Ca2+-tuning signaling molecules not only shapes the kinetics of the Ca2+ flux, but also that of Ca2+- and DAG-sensitive signaling cascades, such as the NF-AT, NF-κB, or MAP kinase pathways, in B cells. Eventually, the combined activities of these pathways adjust the cellular activation and differentiation processes and, thus, control the strength and quality of a humoral immune response [12, 17].

Although the molecular composition of the BCR signalosome as well as the order of events that lead to Ca2+ mobilization have been studied in considerable detail, the picture remains incomplete. Genetic mouse models showed that members of the Vav family of guanine nucleotide exchange factors (GEFs), which control downstream activation of small G proteins of the Rho/Rac family [18], are moreover essential for BCR- (and TCR-) induced Ca2+ mobilization. Whereas solitary deficiency for Vav1 had little impact on BCR-induced Ca2+ signaling, deficiency for Vav1 and Vav2 or all three Vav family members severely impaired Ca2+ signaling in mouse B cells [19–21]. Similarly, a Vav3-deficient variant of the chicken B-cell line DG75 showed reduced BCR-induced Ca2+ signaling and impaired activation of PI3 kinase [22]. Yet, the mechanistic basis for these observations remains unknown. Specifically, it remains unclear if the catalytic GEF activity of Vav proteins is required for BCR-proximal Ca2+ signaling or if Vav proteins fulfill some kind of adaptor function [23]. Moreover, Vav was shown to associate with tyrosine-phosphorylated CD19 and to be required for CD19-mediated amplification of BCR-induced Ca2+ signaling [24]. However, it remains unclear how Vav is employed by the BCR in the absence of CD19 stimulation.

Using a human B-cell model system that was made deficient for Vav1 by genome editing and used for subsequent expression of distinct Vav isoform variants, we show that Vav1 and Vav3 coordinate BCR-induced Ca2+ mobilization via their catalytic activity and by additional means that probably involve adaptor functions. We furthermore show that the GEF activity of Vav is essential for BCR-induced actin reorganization, a process that is supported by all three members of the Vav family, which however, is uncoupled from Ca2+ mobilization. Furthermore, Vav proteins mediate activation of the PI3 kinase/Akt pathway in antigen-activated B cells, which only partially depends on their GEF activity. Mechanistically, Vav1 is directly recruited to the activated BCR and its signalosome by binding to phosphorylated Igα tyrosine residues and to phospho-SLP65. Also, the substrates of Vav family GEFs, small G proteins of the Rho/Rac family, were required for optimal Ca2+ signaling in human B cells. Collectively, our data show that BCR-proximal signaling is critically and differentially coordinated by GEF of the Vav family.

Results
Expression of Vav1 in a human B-cell model system controls BCR-induced Ca2+ mobilization

To investigate the functional principles of Vav family proteins in BCR-induced signaling reactions, we generated a cellular genetic model system by inactivating the VAV1 gene in the human B-cell line DG75 using TALEN-mediated genome editing (for details see Supporting information Fig. S1 and reference [25]). Several Vav1-deficient DG75 subclones showed severely compromised BCR-induced Ca2+ mobilization and, thus, mirrored the phenotype of primary mouse B-cells deficient for either Vav1 and Vav2 or all three Vav isoforms (Fig. 1A). Re-expression of citrine-tagged Vav1 restored the Ca2+ mobilization defect, demonstrating that impaired BCR signaling in Vav1-deficient cells was indeed caused by the lack of Vav1 (Fig. 1B and C). However, the Ca2+ kinetics of cells expressing ectopic Vav1 was enhanced in comparison to parental DG75 cells. Comparison of endogenously and ectopically expressed Vav1 showed that the latter was present in larger amounts (Supporting information Fig. S2A). Consistently, ectopic overexpression of Vav1 in parental DG75 cells enhanced BCR-induced Ca2+ mobilization (Supporting information Fig. S2B and C), indicating that Vav1 regulates the intensity of BCR-proximal signaling in a dose-dependent manner. We then tested if the impaired BCR-induced Ca2+ signal in Vav1-deficient DG75 cells was due to impaired phosphorylation of components of the B cell Ca2+ initiation complex. Thus, we affinity-purified PLCγ2 from lysates of BCR-stimulated B cells, and tested tyrosine-phosphorylation of PLCγ2 and associated proteins by immunoblotting. However, neither tyrosine phosphorylation of PLCγ2 nor that of copurified SLP65 or Igα/β was reduced in cells lacking Vav1 (Fig. 1D). Furthermore, analysis of SLP65 tyrosine phosphorylation by intracellular flow cytometry did not reveal any differences between Vav1-deficient and -proficient cells (Fig. 1E). Thus, Vav1...
does not seem to control BCR-induced Ca\textsuperscript{2+} signaling by facilitating formation of the Ca\textsuperscript{2+} initiation complex. Since Vav1 in B cells is well known for its role in activating MAP kinases [26], we tested whether in the DG75 B cell model system the phosphorylation of Erk and p38 relies on the expression of Vav1. The activation of both MAP kinase pathways was reduced to some extent in Vav1-deficient DG75 B cells, which was restored in cells expressing ectopic Vav1 (Supporting information Fig. 2D-E). Together, these experiments show that Vav1-deficient DG75 cells are a suitable model system to study the mechanics of Vav family proteins in the BCR signaling pathway.

The catalytic activity of Vav is required for BCR-induced Ca\textsuperscript{2+} signaling

Vav family proteins share a common domain architecture, with an N-terminal array of domains that exert and regulate their catalytic GEF activity and a C-terminal cluster of protein-protein interaction domains (see Supporting information Fig. 1F and 2A) [27, 28]. To test the role of the catalytic activity of Vav1 for BCR-proximal signaling, we introduced several amino acid substitutions into two N-terminal domains (Fig. 2A). The glutamate at position 201 within the catalytic Dbl homology (DH) domain of Vav1 forms

Figure 1. (A) Kinetics of BCR-induced Ca\textsuperscript{2+} mobilization of parental DG75 B cells (black curve) and four independent subclones of DG75 in which the VAV1 gene had been inactivated by TALEN-mediated genome editing (see Supporting information Fig. 1 for details). After recording baseline Ca\textsuperscript{2+} levels for 30 s, all cells were stimulated with 20 \mu g/mL of an antihuman IgM F(ab\textsuperscript{2}) fragment (indicated by an upward arrow) and monitored for another 270 s. (B) Ca\textsuperscript{2+} mobilization kinetics of DG75 B cells that were either deficient for Vav1 (Vav1-ko #2, black curve) or reconstituted to express a citrine-tagged variant of Vav1 (Vav1-ko + Vav1, blue curve) using a retroviral expression system. Parental DG75 cells served as control (wt, green line). (C) Citrine fluorescence of cells analyzed in (B). The mean fluorescence intensities (MFI) of the citrine signals are given. (A-C) Data were recorded by flow cytometry. (D) Immunoblot analysis of purified PLC\textgamma\textsubscript{2} and associated proteins from parental DG75 B cells (wt), cells lacking Vav1 (Vav1-ko) and Vav1-ko cells reconstituted with citrine-tagged Vav1 (+ Vav1-Cit). Purified proteins were sequentially analyzed with antibodies to phosphotyrosine (\alpha-pTyr, upper panel), PLC\textgamma\textsubscript{2} as loading control (middle panel), and SLP65 (lower panel). (E) Flow cytometric analysis of tyrosine-phosphorylated SLP65 from DG75 B cells lacking Vav1 (Vav1-ko) or expressing citrine-tagged Vav1 (+ Vav1-Cit). Cells were left untreated (0) or were stimulated with 20 \mu g/mL of an antihuman IgM F(ab\textsuperscript{2}) fragment for 3 min prior to fixation, permeabilization, and staining with antiphospho-SLP65(Y84) coupled to AF-647. The mean fluorescence intensity (MFI) of unstimulated Vav1-ko cells was set to 1.0 in each individual experiment. All other MFIs were normalized accordingly. Error bars indicate standard deviation of three biological replicates with one sample/experiment (n = 3). Statistical analysis was done using two-way ANOVA followed by Tukey’s multiple comparison test. ns: not significant. Data from (A) are representative of two and data from (B), (C), and (D) are representative of three biological replicates with one sample per experimental condition (n = 3).
resulted in markedly reduced BCR-induced Ca\(^{2+}\). Corresponding alanine replacements (Q331A, N371A, and E378A) also suggested that position 378 of Vav1 make contacts with Rac1 [29, 30]. The corresponding alanine replacements (Q331A, N371A, and E378A) also suggested that position 378 of Vav1 make contacts with Rac1 [29, 30]. The corresponding alanine replacements (Q331A, N371A, and E378A) also suggested that position 378 of Vav1 make contacts with Rac1 [29, 30]. The corresponding alanine replacements (Q331A, N371A, and E378A) also suggested that position 378 of Vav1 make contacts with Rac1 [29, 30].

The zinc finger (ZF) of Vav1 makes intramolecular contacts with Rac1 [29]. Replacement of this glutamate with alanine (E201A) was reported to eliminate Vav1 GEF activity [29], which we confirmed in vitro GEF assays toward the small G proteins Rac1, RhoA, and Cdc42 (Supporting information Fig. S4F). Importantly, all of the analyzed DH domain mutants were expressed in amounts comparable to WT Vav1 (Fig. 2C and Supporting information Fig. S4B, D, & G).

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in our in vitro GEF assays toward Rac1 and RhoA (Supporting information Fig. S3). Expression of a Vav1 variant lacking the AR (Vav1-ΔAR) resulted in a markedly enhanced Ca\textsuperscript{2+} profile in BCR-activated DG75 cells (Fig. 2F), even though this variant was less well expressed than WT Vav1 (Fig. 2G). Together, these experiments reveal a critical function of the catalytic GEF activity of Vav1 for optimal BCR-induced Ca\textsuperscript{2+} signaling, and thus, show that Vav1 does not just act as an adaptor protein in that process.

**Vav1 is directly recruited to the phosphorylated BCR**

Having established the importance of the catalytic activity of Vav1 for BCR-induced Ca\textsuperscript{2+} signaling, we tested the role of its C-terminal adapter region for that process. We first inactivated the Vav1 SH2 domain by replacing the critical arginine residue in the SH2 domain binding pocket with alanine (R696A), which completely suppressed Vav1’s function in the BCR signaling cascade (Fig. 3A and B). To identify the Vav1 SH2 domain interaction partners within the BCR signalosome, we performed affinity purification experiments using a recombinant Vav1 SH2 domain and lysates of BCR-activated B cells. As controls, we used the R696A-mutant SH2 domain and the tandem SH2 domains of the PTK Syk. Immunoblotting with anti-phosphotyrosine antibodies showed that the WT, yet not the R696A-mutant Vav1 SH2 domain interacts with a distinct set of phosphoproteins from activated B cells (Fig. 3C). The higher molecular weight proteins were identified as SLP65, Syk, and HS1 in separate experiments (Supporting information Fig. S5 and data not shown). Strikingly, the Vav1 SH2 domain purified a diffusely running set of phosphoproteins that was also purified by the tandem SH2 domains of Syk, which are well known to bind to the I\(\alpha\)\(\beta\) heterodimer of the BCR. We identified these proteins as I\(\alpha\)\(\beta\) by direct immunoblotting (Supporting information Fig. S5A). To further characterize the interaction between Vav1 and the BCR, we used a set of phosphorylated peptides encompassing the known tyrosine-phosphorylation motifs of I\(\alpha\) and I\(\beta\), respectively, for affinity purification experiments from B cell lysates (Supporting information Fig. S5B). Indeed, Vav1 interacted with phosphopeptides from I\(\alpha\) in which either the ITAM tyrosines were phosphorylated (Fig. 3D, I\(\alpha\)-pepI 1 and 1+2) or in which the non-ITAM tyrosine residue Y204 was phosphorylated (I\(\alpha\)-pepII 2+3 and 3). However, Vav1 was not purified by the phosphorylated I\(\beta\) peptide, indicating that the SH2 domain of Vav1 prefers the phosphotyrosine binding motifs of I\(\alpha\) over those of I\(\beta\). To verify the integrity of the used phosphopeptides, we tested for the presence of the known BCR-interacting proteins Syk and SLP65, which were bound by either doubly-phosphorylated ITAM motifs (Syk) or the I\(\alpha\) non-ITAM Y204 motif (SLP65) as expected (Fig. 3D, middle and lower panel, respectively). Finally, binding of a recombinant Vav1 SH2 domain to a phosphopeptide from I\(\alpha\) proved that complex formation between Vav and phospho-I\(\alpha\) is due to a direct interaction of the two partners (Fig. 3E).

**Complex formation between Vav1 and the BCR facilitates Ca\textsuperscript{2+} signaling**

To investigate whether the newly discovered interaction between Vav1 and I\(\alpha\) tyrosine docking sites in the BCR is of functional relevance, we generated chimeric variants of Vav1 in which we replaced its endogenous SH2 domain with either those of Syk or that of SLP65 (see Supporting information Fig. S6A). The SH2 domains of Syk and SLP65 specifically bind to either ITAM or non-ITAM tyrosine motifs of the BCR (see Fig. 3D) and, hence, target the chimeric Vav1 variants directly to the activated BCR (Supporting information Fig. 6B). Indeed, both Vav1-SH2 domain chimeras restored BCR-induced Ca\textsuperscript{2+} mobilization albeit to different extents (Fig. 4A and Supporting information Fig. S6C). These differences are most likely due to diverging affinities and numbers of available binding sites (one non-ITAM tyrosine (Y204) motif for the SLP65 SH2 domain versus two ITAM motifs for the tandem SH2 domains of Syk per BCR complex, see Supporting information Fig. S6D). Note that also the Vav1 SH2 domain has several potential docking sites per BCR complex and furthermore can interact with SLP65 and Syk (see Fig. 2), which might explain why it works better than the SH2 domain of SLP65. To exclude that the SLP65 SH2 domain in the context of the chimeric Vav1 protein uses an unknown alternative binding site besides I\(\alpha\) Y204, we additionally expressed a chimeric transmembrane protein consisting of the extracellular and transmembrane parts of CD8 and the intracellular domain of I\(\alpha\) in cells expressing either WT Vav1 or the Vav1-SLP65 SH2 domain chimera (Supporting information Fig. S6D). Stimulation of this CD8/I\(\alpha\) chimera resulted in a BCR-like Ca\textsuperscript{2+} signal in cells expressing WT Vav1 and a slightly reduced signal in cells expressing the Vav1-SLP65 SH2 domain chimera, while inactivation of the SLP65 SH2 domain (Vav1-SLP65-SH2-RL) disabled Ca\textsuperscript{2+} signaling (Fig. 4B). Likewise, inactivation of Y204 in the CD8/I\(\alpha\) chimera receptor (CD8/I\(\alpha\)Y204F) selectively abolished Ca\textsuperscript{2+} mobilization in cells expressing the Vav1-SLP65 SH2 domain chimera, but not in cells expressing WT Vav1 (Fig. 4C). These results show that the SH2 domains of Syk and SLP65 targeted the Vav1 chimeras to the phosphorylated BCR, and that this route of Vav1 plasma membrane recruitment is functionally relevant for BCR-induced Ca\textsuperscript{2+} mobilization.

**Binding of Vav to phospho-SLP65 represents an alternative membrane recruitment pathway**

Previous studies had shown that Vav1 can associate via its SH2 domain with phosphorylated SLP65 [7, 34], but a functional role of this protein-protein interaction was never described. Hence, we tested whether this interaction is of functional relevance using a similar approach as before. This time we replaced the Vav1 SH2 domain with those of the Tec family PTKs Btk or Itk (Supporting information Fig. S6E), both of which interact with one specific tyrosine-phosphorylation site, Y96, in human SLP65 [7, 8] (Supporting information Fig. S6F). Noteworthy, the SH2 domain of Itk has a superior affinity compared to that of Btk (Supporting information Fig. S6G).
information Fig. 6G) as previously reported [8]. Both SH2 domain chimeras could support BCR-induced Ca²⁺ mobilization to some extent (Fig. 4D), showing that the recruitment of Vav1 to phospho-SLP65, that is, to the immediate microenvironment of activated BCRs, is sufficient to allow Vav proteins to exert their function in BCR-proximal signaling reactions.

Distinct Vav family members differentially support BCR-induced Ca²⁺ signaling

So far, it remained unclear whether different Vav family members serve redundant or unique functions in B cells. Thus, we tested the expression of all three Vav family members in primary
Vav proteins stimulate BCR-induced activation of the PI3 kinase/Akt pathway

A previous report suggested a role for Vav3 in the activation of PI3 kinase in the chicken B-cell line DT40, yet the mechanistic basis for this observation remained unclear [22]. Hence, we used our human B-cell model system to address that issue. As readout for PI3 kinase activity, we measured phosphorylation of the serine/threonine kinase Akt (PKB) by intracellular flow cytometry. Indeed, BCR-induced phosphorylation of Akt was reduced in Vav1-deficient B cells to about 50% of the level observed in parental DG75 cells (Fig. 6A and Supporting information Fig. S8). This defect was restored by re-expression of WT Vav1, and also by expression of the catalytically inactive Vav1 E201A variant, albeit to a somewhat reduced extent (Fig. 6A), indicating that Vav-mediated PI3 kinase activation is partially uncoupled from its GEF activity. Like Vav1, also Vav3 fully supported activation of Akt (Fig. 6B).

Since the magnitude of BCR-induced Ca\(^{2+}\) mobilization roughly correlated with the efficiency of Akt phosphorylation in our experiments, we tested whether Ca\(^{2+}\) mobilization, is required for PI3 kinase activation in B cells. For this purpose, we used DG75 B cells deficient for both PLC\(\gamma\) isoforms, PLC\(\gamma\)1 and PLC\(\gamma\)2 (PLC\(\gamma\)-dko) that are completely deficient for BCR-induced Ca\(^{2+}\) signaling [25]. More precisely, we tested in this experiment if any of the PLC\(\gamma\)-derived second messengers controls PI3 kinase signaling. Figure 6C shows that BCR-induced Akt phosphorylation did not

Human peripheral blood B cells and in our DG75 B cell model system (Fig. 5A). All three isoforms of Vav were readily detected in lysates of primary human B cells. By contrast, DG75 B cells expressed considerable amounts of Vav1 and Vav2, but hardly any Vav3. When expressed in Vav1-deficient DG75 B cells, all three Vav family members differed strongly in their ability to promote BCR-induced Ca\(^{2+}\) signaling. Whereas expression of Vav3 in DG75 cells caused a much enhanced and strongly prolonged Ca\(^{2+}\) signal as compared to expression of Vav1, expression of Vav2 did not restore BCR-induced Ca\(^{2+}\) mobilization at all (Fig. 5B). Notably, Vav3 was a very strong stimulator of Ca\(^{2+}\) mobilization despite being expressed at reduced amounts compared to Vav1 and Vav2 (Fig. 5C). To test if the robust Ca\(^{2+}\) signal caused by Vav3 required its GEF activity, we inactivated the catalytic DH domain in Vav3 by replacing the glutamate at position 199 with alanine (E199A, Supporting information Fig. S7). Similar to the Vav1 E201A variant, E199A-mutant Vav3 gave rise to a strongly reduced Ca\(^{2+}\) signal (Fig. 5D), even though its expression level was comparable to that of WT Vav3 (Fig. 5E).

We furthermore investigated why Vav2 failed to support BCR-induced Ca\(^{2+}\) signaling. We hypothesized that its inhibitory AR might interfere with proper activation of this isoform in B cells. To test this notion, we deleted the AR in Vav2 (Vav2-ΔAR) and tested its functionality as before (Fig. 5F). Even though Vav2-ΔAR was less well expressed than WT Vav2 (Fig. 5G), it fully restored Ca\(^{2+}\) signaling, indicating that Vav2 can mediate Ca\(^{2+}\) mobilization in B cells when its catalytic activity is liberated.

Figure 4. (A) BCR-induced Ca\(^{2+}\) kinetics of Vav1-deficient DG75 cells expressing either citrine-tagged WT Vav1 (blue curve) or chimeric Vav1 variants containing the SH2 domain of SLP65 (Vav1-SLP65-SH2, red curve) or the tandem SH2 domains of Syk (Vav1-Syk-[SH2], pink curve), respectively. Vav1-deficient cells expressing EGFP served as control (EGFP, black curve). Cells were stimulated with 20 μg/mL of an anti-human IgM F(\(\alpha\)\(\beta\))\(\gamma\) fragment (indicated by an upward arrow). (B) Vav1-deficient cells reconstituted with either WT citrine-tagged Vav1 (Vav1, blue curve) or Vav1-chimeras containing the SH2 domain of SLP65 (Vav1-SLP65-SH2, red curve) or an SH2 domain-inactive variant thereof (Vav1-SLP65-SH2-RL) were additionally equipped with a chimeric CD8 molecule containing the intracellular domain of Ig\(\alpha\) (CD8-Ig\(\alpha\)). Ca\(^{2+}\) mobilization in the cells was monitored following stimulation with 10 μg/mL anti-CD8 antibodies (indicated by an upright arrow). (C) The same cells as before were equipped with a CD8/\(\mu\)g-chimera in which the non-ITAM tyrosine residue 204 was replaced with phenylalanine (CD8-\(\mu\)gY204F) and analyzed as in (B). (D) Vav1-deficient cells expressing chimeric variants of Vav1 containing the SH2 domains of either Btk or Itk were analyzed as in (A).

All data are representative of three biological replicates (n = 3) with one sample of each cell type per experiment and were measured by flow cytometry.
require expression of PLCγ. Hence, the Ca2+-promoting activity of Vav1 and Vav3 is not directly responsible for PI3 kinase signaling in BCR-activated cells.

We furthermore tested the ability of Vav2 to promote activation of PI3 kinase. Surprisingly, BCR-induced phosphorylation of Akt could be restored by expression of Vav2 (Fig. 6D) although this isoform failed to support Ca2+-signaling (see Fig. 5B). This result indicates that insufficient activation of PI3 kinase in the Vav1-deficient cells is not responsible for defective Ca2+-signaling. Notably, the level of Akt phosphorylation in cells expressing Vav2 resembled that of cells expressing catalytically inactive Vav1. Thus, we tested if deletion of the AR in Vav2 would further enhance activation of Akt and found that the ΔAR-variant of Vav2 indeed had a somewhat increased ability to promote phosphorylation of Akt (Fig. 6D). In summary, these results demonstrate that distinct members of the Vav GEF family differentially control BCR-induced Ca2+ mobilization and activation of the PI3 kinase/Akt pathway.

Vav proteins are a branching point for BCR-induced Actin reorganization and Ca2+ mobilization

Reorganization of the actin cytoskeleton has been implicated to be involved in the initiation of BCR-induced Ca2+ mobilization [14, 15]. Since Vav proteins are activators of Rho/Rac family small G proteins, which control Actin reorganization in many cell types, we tested whether they control actin remodeling downstream of the BCR (Supporting information Fig. S9). Cells expressing WT Vav1 showed an increased Phalloidin staining intensity that peaked after 30 to 60 s following BCR activation and reached a plateau between the 3 and 7 min stimulation time points (Fig. 7A, blue curve). By contrast, BCR-induced formation of F-Actin was severely compromised in cells lacking Vav1 (Fig. 7A, black curve). Catalytically inactive Vav1-E201A was impaired in its ability to support BCR-induced Actin remodeling (Fig. 7B), indicating that the GEF activity of Vav is required for that process. In line with

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Figure 6. (A) Analysis of Akt phosphorylation on serine 473 in parental DG75 B cells (DG75), cells lacking Vav1 (Vav1-ko) or Vav1-ko cells expressing either citrine-tagged WT Vav1 (+Vav1-Cit) or E201A-mutant Vav1 (+Vav1-Cit-E201A). Cells were either left untreated (0) or stimulated with anti-human IgM F(ab')2 fragments for 3 min (3), followed by fixation, permeabilization, intracellular staining, and flow cytometric analysis. The mean fluorescence intensity (MFI) of unstimulated Vav1-ko cells was set to 1.0 in each individual experiment. All other MFIs were normalized to their corresponding reference value. Error bars indicate mean ± SD of three independent experiments. Statistical analysis was done using two-way ANOVA followed by Tukey’s multiple comparison test. ****p < 0.0001. (B) Analysis of phospho-Akt was done as in (A) with DG75 cells, Vav1-ko cells, and Vav1-ko cells expressing citrine-tagged Vav3. (C) Phospho-Akt was analyzed as in (A) in parental DG75 cells and cells lacking either Vav1 or both isoforms of PLCγ (PLCγ1/2-ko). (D) Phosphorylation of Akt in DG75 B cells that were deficient for Vav1 (Vav1-ko) or expressed WT citrine-tagged Vav2 (+Vav2-Cit) or a variant of Vav2 lacking the acidic region (+Vav2-Cit-ΔAR). Analysis was done as in (A). All data show mean fluorescence intensities of three biological replicates (n = 3) with one sample of each condition per experiment.

our previous observations regarding Ca^{2+} mobilization, Vav3 was even more effective than Vav1 in actin remodeling despite being less well expressed (Figs. 5C and 7C). Surprisingly, also Vav2 was a very effective mediator of BCR-induced actin reorganization (Fig. 6D). This result indicated that BCR-induced reorganization of the actin cytoskeleton and the mobilization of Ca^{2+} ions are separable events. To test this notion in more detail, we titrated the Actin polymerization-inhibiting drug Latrunculin A (LatA) to determine the minimal effective concentration that was needed to prevent formation of F-Akt in BCR-activated DG75 cells (Supporting information Fig. S10). We then pretreated Vav1-expressing B cells for 5 min (as we did in the titration experiments shown in Supporting information Fig. S10) with concentrations of either 0.5 or 1 μM LatA to block actin reorganization, and then analyzed BCR-induced Ca^{2+} mobilization. Evidently, inhibition of actin reorganization had no detectable effect on BCR-induced Ca^{2+} signaling (Fig. 7E). However, since administration of LatA to B cells was previously reported to instantly evoke a BCR-like Ca^{2+} signal [15], we repeated the experiment and this time monitored Ca^{2+} flux during the 5 min incubation time with LatA, followed by BCR stimulation as before. Under these conditions, LatA caused a detectable, yet very subtle release of Ca^{2+}, which was strongly enhanced by BCR stimulation (Fig. 7F). In conclusion, Vav family proteins are critical components for both
Since our experiments revealed that the catalytic GEF activity of Vav family substrates promotes BCR-induced Ca\textsuperscript{2+} signaling (Vav1-Cit), cells were either left untreated (0) or were stimulated with antihuman IgM F(ab')\textsubscript{2} fragments for the indicated time points. Subsequently, cells were fixed, permeabilized, and stained with AlexaFluor647-conjugated Phalloidin. Mean fluorescence intensities (MFI) were determined by flow cytometry. The MFI of unstimulated Vav1-ko cells was set to 1.0 in each individual experiment. All other MFIs were normalized to their corresponding reference value. Error bars indicate mean ± SD of three experiments. Statistical analyses were done using two-way ANOVA followed by Sidak’s multiple comparison tests. ***p < 0.001, **p < 0.01, *p < 0.05, ns: not significant.

| Figure 7 | **A** Kinetics of F-Actin formation in BCR-stimulated DG75 B cells either lacking Vav1 (Vav1-ko) or expressing citrine-tagged WT Vav1 (Vav1-Cit). Cells were either left untreated (0) or were stimulated with antihuman IgM F(ab')\textsubscript{2} fragments for the indicated time points. Subsequently, cells were fixed, permeabilized, and stained with AlexaFluor647-conjugated Phalloidin. Mean fluorescence intensities (MFI) were determined by flow cytometry. The MFI of unstimulated Vav1-ko cells was set to 1.0 in each individual experiment. All other MFIs were normalized to their corresponding reference value. Error bars indicate mean ± SD of three experiments. Statistical analyses were done using two-way ANOVA followed by Sidak’s multiple comparison tests. ***p < 0.001, **p < 0.01, *p < 0.05, ns: not significant. **B** Same analysis as in (A) with cells expressing the E201A-mutant variant of Vav1. (C) Same analysis as in (A) with cells expressing citrine-tagged Vav3. (D) Same analysis as in (A) with cells expressing citrine-tagged Vav2. (E) BCR-induced Ca\textsuperscript{2+} mobilization kinetics of Vav1-deficient DG75 cells expressing citrine-tagged WT Vav1 preincubated for 5 min either with DMSO (blue curve) or 0.5 μM (red curve) or 1 μM (black curve) Latrunculin A (LatA) before stimulation with 20 μg/mL of an antihuman IgM F(ab')\textsubscript{2} fragment (indicated by an upward arrow). (F) Same analysis as in (E) including the 5 min preincubation time with the indicated reagents. (A-D) show mean fluorescence intensities of three biological replicates (n = 3) with one sample of each condition per experiment, (E) & (F) are representative of three biological replicates (n = 3) with one sample of each condition per experiment.

BCR-induced Ca\textsuperscript{2+} mobilization and actin reorganization, yet they appear to represent branching points at which these two processes diverge.

**Vav family substrates promote BCR-induced Ca\textsuperscript{2+} signaling**

Since our experiments revealed that the catalytic GEF activity of Vav proteins regulates BCR-proximal Ca\textsuperscript{2+} mobilization, we tested whether the substrates of Vav, small G proteins of the Rho/Rac family, are involved in that process as well. Recently, two effective pharmacological inhibitors of Rho/Rac proteins were described, called Rhosin and EHop-016, respectively [35, 36]. Rhosin is reported to be specific for RhoA whereas EHop-016 seems to preferentially target Rac proteins. Both inhibitors strongly impaired Ca\textsuperscript{2+} signaling in DG75 B cells, resulting in Ca\textsuperscript{2+} kinetics that resembled that of Vav1-deficient cells (Fig. 8A). Treatment of primary human B cells with EHop-016 caused a similar reduction in BCR-induced Ca\textsuperscript{2+} signaling as in DG75 B cells (Fig. 8B & Supporting information Fig. S11), indicating that Rac and Rho family proteins are indeed involved in Ca\textsuperscript{2+} signaling in antigen-activated B cells. To verify the inhibitor experiments and to more precisely identify the small G protein(s) involved in BCR-induced Ca\textsuperscript{2+} mobilization, we furthermore generated genetic model systems. Since inactivation of Rac2 in the mouse was previously reported to affect Ca\textsuperscript{2+} mobilization in B cells [37], we first deleted Rac2 in DG75 B cells using CRISPR/Cas9-directed genome editing (Supporting information Fig. S12A-D). Similar to mouse B cells, deficiency for Rac2 had a moderate effect on BCR-induced Ca\textsuperscript{2+} mobilization in DG75 cells (Fig. 8C). As controls, we ectopically expressed Rac2 in the Rac2-ko cells and in parental DG75
cells (Supporting information Fig. S12E), which rescued the Ca²⁺ mobilization defect of Rac2-deficient cells (Fig. 8D) and resulted in an elevated Ca²⁺ signal in parental DG75 B cells, respectively (Supporting information Fig. S12F). Nevertheless, since Rac2-deficiency did not fully recapitulate the Ca²⁺ mobilization defect of DG75 cells expressing catalytically inactive Vav1 or Vav3, we furthermore inactivated Rac1 by CRISPR/Cas9-mediated genome editing (Supporting information Fig. S13A-C). Deficiency for Rac1 resulted in a noticeable reduction of BCR-induced Ca²⁺ mobilization (Fig. 8E). Again, ectopic re-expression of Rac1 reverted the Ca²⁺ phenotype of the Rac1-ko cells (Supporting information Fig. 13D and Fig. 8F). We also tried to generated Rac1/2 double-deficient DG75B cells and to inactivate RhoA by CRISPR/Cas9 mutagenesis, however, neither Rac1/2 double-deficient cells nor RhoA-deficient clones could be obtained, probably because DG75 cells require these proteins for survival and/or proliferation. Hence, we could not test the role of RhoA in BCR-proximal Ca²⁺ signaling in a genetic model system. Nevertheless, the available
genetic and pharmacological data indicate that probably both, Rac and RhoA, are involved in BCR-proximal second messenger generation. Rac1 and Rac2 have previously been implicated in the activation of PLCγ in BCR-activated chicken B cells [38]. This mechanism seems to involve a tyrosine-phosphorylation-independent protein-protein interaction between Rac and PLCγ2 [39], which can be abrogated by replacing phenylalanine 897 of PLCγ2 with glutamine (F897Q) [38]. To test whether the missing interaction between Rac and PLCγ2 could explain the Ca2⁺ phenotype of DG75 cells lacking Rac1 or Rac2, we tested the performance of F897Q-mutant PLCγ2 (tagged with EGFP) in PLCγ2-deficient DG75 B cells. As controls, we analyzed cells expressing WT PLCγ2 and cells lacking Rac2. Indeed, cells expressing F897Q-mutant PLCγ2 showed a reduced Ca2⁺ kinetics that was even a little lower than that of Rac2-deficient cells (Fig. 8G), which may indicate that Rac2 and Rac1 are redundant in their ability to activate PLCγ2. Importantly, WT and F897Q-mutant PLCγ2 were expressed in equal amounts in the transfected cells (Fig. 8H).

Discussion

Here, we have shown that Vav proteins regulate critical BCR signaling processes including mobilization of the key second messenger Ca2⁺, activation of the PI3 kinase/Akt pathway, and actin cytoskeleton remodeling in the human Burkitt lymphoma cell line DG75. The involvement of Vav isoforms in B-cell Ca2⁺ signaling was observed earlier in gene-targeted mice lacking more than one Vav family member. However, these previous results lacked a mechanistic explanation and furthermore were somewhat inconsistent. Whereas some groups reported that BCR-induced Ca2⁺ signaling is normal in cells lacking only Vav1 but defective in B cells lacking Vav1 and Vav2 [19, 20] others found essentially normal Ca2⁺ mobilization in B cells from the very same Vav1/2 double-deficient mice [15]. In addition, another group showed defective Ca2⁺ signaling in mouse B cells lacking all three Vav family members [21]. Our human Vav1-deficient B cells expressed only low amounts of Vav2 and hardly any Vav3. They thus represent a suitable model system to study distinct Vav isoforms. Whereas Vav1 and Vav3 were able to promote B cell Ca2⁺ signaling, Vav2 failed to support this process. This was not due to a general functional defect of this isoform, since Vav2 efficiently promoted BCR-induced activation of PI3 kinase/Akt as well as cytoskeletal reorganization. Interestingly, also in Jurkat T cells, Vav2 failed to support TCR-induced Ca2⁺ mobilization [40]. By contrast, murine and chicken Vav2 were previously shown to facilitate BCR-induced Ca2⁺ mobilization in a murine and in a chicken B cell line, respectively [22, 41]. This discrepancy to our results may be due to species-related differences of either the used Vav2 orthologues or the respective cellular context. Indeed, species-dependent differences were recently observed with regard to activation of the Erk MAP kinase pathway in antigen-activated B cells [25]. Whatever the exact reason may be, the inability of human Vav2 to facilitate BCR-induced Ca2⁺ signaling could be relieved by deletion of its inhibitory AR, indicating that this domain effectively and selectively blocks the Ca2⁺-promoting activity of Vav2 [42]. Furthermore, our mutational analyses of the DH domain and the ZF region in Vav1 and Vav3 clearly showed that the catalytic activity of Vav is mandatory for proper BCR-induced Ca2⁺ signaling. However, the Ca2⁺ kinetics of DH domain-mutant Vav1 and Vav3 were not as strongly reduced as that of Vav1-ko cells, indicating that additional Vav-specific mechanisms are required for an optimal Ca2⁺ response, which may involve adaptor functions of either the C-terminal SH3 domains and/or the N-terminal CH domain. Future experiments will be required to reveal the function of these domains in BCR-proximal signaling. The requirement of Vav3 for BCR-induced activation of PI3 kinase/Akt has previously been observed in the chicken B cell line DT40 [22]. This observation led to the conclusion that impaired PI3 kinase activation is responsible for the Ca2⁺ mobilization defect in Vav3 deficient DT40 B cells [22]. However, our results challenge this interpretation, since expression of human Vav2 in DG75 cells restored PI3 kinase signaling, yet completely failed to support Ca2⁺ mobilization. Hence, besides facilitating the activation of PI3 kinase there must be an alternative and/or additional mechanism by which Vav proteins promote Ca2⁺ signaling in B cells. We tested whether this alternative mechanism involves reorganization of the Actin cytoskeleton, since previous studies had inferred that the actin membrane skeleton controls BCR activation [14, 15]. Indeed, BCR-induced formation of F-Actin was strongly reduced in Vav1-deficient DG75 B cells and — like the Ca2⁺ mobilization kinetics — could only partially be restored by catalytically inactive E201A-mutant Vav1. Similar to the activation of PI3 kinase, Actin remodeling was very efficiently restored by Vav2, which however, failed to promote Ca2⁺ mobilization. Furthermore, pharmacological inhibition of Actin reorganization by Latrunculin A treatment had hardly any detectable effect on Ca2⁺ signaling in B cells. These findings combined make it unlikely that the Actin cytoskeleton is a key control element of BCR-proximal Ca2⁺ signaling. Consistently, a recent report showed that Latrunculin A-induced Ca2⁺ signaling is specific for IgD-containing BCRs and furthermore requires coexpression of CD19 and the chemokine receptor CXCR4 [16]. Taken together, Vav proteins are apparently involved in the activation of several distinct signaling processes that are initiated by BCR engagement, yet these distinct signaling reactions appear to be — at least partially — uncoupled from each other. Thus, Vav proteins seem to serve as branching points in the BCR signaling cascade (Supporting information Fig. S14).

It is important to note that we have used soluble reagents for BCR stimulation in our experiments. However, B cells can as well detect cell surface-bound antigens, in which case the Actin cytoskeleton plays a central role when B cells spread over membranes and use mechanical force to actively take up antigen [43–46]. Yet the factors that couple BCR signaling to reorganization of the actin cytoskeleton during recognition of membrane-bound antigen are not well characterized. Our data suggest that Vav proteins may be key mediators in that process.
Since the catalytic activity of Vav was required for proper Ca\textsuperscript{2+} signaling in B cells, we tested the involvement of small G proteins of the Rho/Rac family using two approaches: pharmacological inhibition and genetic inactivation. The RhoA inhibitor Rhosin (which may also target additional closely related Rho family members) strongly impaired BCR-induced Ca\textsuperscript{2+} mobilization. Pharmacological inhibition of Rac proteins using the inhibitor EH-016 had a similar effect. EH-016 probably inhibits several Rac isoforms, leaving unclear precisely which one is employed in BCR-proximal signaling. However, since pharmacological inhibitors always bear the risk of causing nonspecific side effects, we furthermore performed genetic experiments. Genetic inactivation of either Rac1 or Rac2 caused a reduction of BCR-induced Ca\textsuperscript{2+} mobilization, which however was less pronounced than that caused by EH-016. This indicates that both Rac isoforms have partially redundant functions. Unfortunately, this could not be tested in DG75 cells since Rac1/2 double-deficient cells (like RhoA deficient cells) could not be obtained. More recently, even Cdc42 has been implicated to regulate BCR-proximal signaling [47]. However, in our in vitro GEF assays Vav was not a very robust activator of Cdc42. While Rho/Rac proteins have been widely described as regulators of the actin cytoskeleton [48], their exact functions in BCR-proximal Ca\textsuperscript{2+} signaling and PI3 kinase activation remains to be clarified in future studies. Notably, RhoA, Rac, and Vav proteins have all been implicated in the activation of phosphatidylinositol 4-phosphate 5-kinases, which generate phosphatidylinositol-4,5-bisphosphate (PIP2), the substrate for PLC\textgamma\gamma[24, 49, 50]. This scenario could explain why PLC\textgamma\gamma phosphorylation was normal in Vav1-deficient cells, since it would affect the output of PLC\textgamma\gamma but not its activation. In addition, Rac proteins were reported to directly activate PLC\textgamma\gamma via a protein-protein interaction, which is prevented in the F897Q-mutant variant of PLC\textgamma\gamma2 [38, 39]. Indeed, the Ca\textsuperscript{2+} kinetics of cells expressing F897Q-mutant PLC\textgamma\gamma2 closely mirrored those of Rac2-deficient cells, indicating that this mechanism may — at least in part — be responsible for the diminished Ca\textsuperscript{2+} kinetics of Rac-deficient DG75 and mouse B cells [37] and, thus, probably also for that of cells expressing catalytically inactive Vav1 or Vav3. Importantly, the requirement of GTP-bound Rac proteins for optimal phospholipase activation applies to PLC\textgamma\gamma2 but not to PLC\textgamma\gamma1 [51], which may explain why expression of the catalytically inactive LK-AA-mutant Vav1 in T cells did not result in diminished TCR-induced Ca\textsuperscript{2+} signaling, since T cells express PLC\textgamma\gamma1 but not PLC\textgamma\gamma2 [31].

We furthermore showed that in B-cells Vav1 needs to be recruited via its SH2 domain to the BCR signalosome. The association between Vav1 and SLP65 (BLNK) was already reported together with the initial identification and characterization of SLP65 [7, 34]. However, the functional significance of this interaction was never tested. Our data demonstrate that the recruitment of Vav1 to phosphorylated SLP65 is indeed of functional importance in BCR-proximal signaling. However, Vav1 also associates directly with the activated BCR and this interaction is of functional relevance as well. In conclusion, Vav proteins probably do not need to be precisely localized at a defined docking site within the BCR signalosome like, for example, Btk, which specifically interacts with one particular tyrosine phosphorylation motif in SLP65 [7, 8], but instead can opt between multiple phosphorylated tyrosine residues, depending on their availability. In conclusion, the differential functional capabilities of Vav family members within the BCR signaling cascade may indicate specialized functions of individual Vav proteins in different B-cell subpopulations and/or different stages of B-cell development and furthermore may allow for pharmacological inhibition of these molecules in the treatment of B cell-related diseases.

**Materials and methods**

**Cells and genome editing**

The human Burkitt lymphoma B-cell lines DG75 and Ramos were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All cells were maintained in RPMI1640 + Glutamax (Biochrom, Berlin, Germany) supplemented with 10 % heat-inactivated FCS and antibiotics. The DG75EB variant of the cells, which expresses the murine cationic amino acid transporter 1 (SLC7A1) to make them susceptible to infection with MMLV-based retrovirus particles as well as the PLC\textgamma2-deficient and the PLC\textgamma1/2 double-deficient sublines of DG75 cells were described before [25, 52]. DG75 cells deficient for Vav1 were generated by TALEN-mediated genome editing. TALEN cassettes were designed using the TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/) and generated using a modified version of the “Golden Gate” TALEN assembly method [53]. The plasmid kit used for generation of TALENs was a gift from Daniel Voytas and Adam Bogdanove (Addgene kit # 1000000024). Modifications included shortening of the linker regions N- and C-terminal of the DNA binding modules and optimization of the FokI nuclease by introducing “Sharkey” mutations that cause a higher activity without increasing off-target effects [25, 54]. The TALEN constructs (designated left and right TALEN, respectively) were cloned into expression vectors pmax-IE and pmax-IR, containing an IRES-EGFP (IE) and an IRES-tagRFP (IR) cassette, respectively. DG75 cells were transiently transfected with both plasmids using the Amaxa Nucleofector\textsuperscript{TM} II device (Lonza, Basel, Switzerland) in combination with the Lonza Human B cell Nucleofector\textsuperscript{TM} Kit (program T-015). Two days after electroporation, EGFP/RFP double-positive cells were sorted, expanded, subcloned, and screened for deficiency of the target protein (see Supporting information Fig. S1 for further details). Vav1-deficient cells were retrovirally transduced to express SLC7A1. The resultant DG75EB Vav1\textsuperscript{−/−} cells were used in all experiments. Single guide RNAs were designed with the Zhang-lab online software (crispr.mit.edu) and were cloned as synthetic oligos into the pSpCas9(BB)-2A-GFP vector (Addgene #48138) [55], followed by Amaza nucleofection, cell sorting for GFP-positive cells, subcloning, and screening by immunoblotting.
Expression vectors and retroviral gene transfer

The cDNAs encoding for human Vav and Rac family members were purchased from Dharamcon (Lafayette, CO, USA). The cDNA encoding rat PLCγ2 was provided by Dr. Tomohiro Kurosaki, Osaka University, Japan, and was equipped with an N-terminal EGFP tag. Chimeric variants of Vav1 were generated using overlap PCR. Chimeric CD8/ Igκ was described previously [56]. For site-directed mutagenesis, cDNAs were cloned into the plasmid pCR2.1 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The integrity of all constructs was confirmed by Sanger DNA sequencing (SeqLab-Microsynth, Göttingen, Germany). For expression, all Vav constructs were C-terminally fused to citrine and cloned into pMScVpuro (Clontech, Takara Bio Inc, Kyoto, Japan). Rac isofroms were expressed using the MIGRII vector containing an IRES-EGFP cassette. Retroviral transductions were done with the packaging cell line Plat-E. Transduced cells were selected in the presence of 2 μg/mL puromycin for 5-7 days followed by expression analysis by flow cytometry and western blotting.

Biochemical assays, antibodies, and reagents

For BCR stimulation, cells were incubated with 20 μg/mL goat antimouse IgM F(ab')2 fragments (Jackson ImmunoResearch, West Grove, PA, USA) at 37°C in RPMI without FBS for the indicated times. Preparation of cellular lysates in 1% NP40-containing lysis buffer for affinity purifications and western blot analyses was done as described [6]. Briefly, the cells were lysed with lysis buffer composed of 50 mM Tris-HCl (pH 7.8), 137 mM NaCl, 0.5 mM EDTA, 1 mM sodium orthovanadate, 10% v/v glycerol, 1% v/v NP40, and a protease inhibitor cocktail containing AEBSF, Aprotinin, Bestatin, E-64, Leupeptin and EDTA (Sigma Aldrich, #P2714). Lysis was performed on ice for 10 min and the cell debris was pelleted at 20,000 rpm for 10 min. The supernatant containing the cell lysate was mixed with reducing SDS-PAGE sample buffer, boiled at 95°C for 5 min and analyzed by SDS-PAGE and immunoblotting. For affinity purification of proteins from B-cell lysates using biotinylated peptides or GST fusion proteins, cell lysates were prepared as described above from 3 × 10^7 Ramos cells and incubated with 2 μM peptides or ~20 μg GST fusion proteins bound to glutathione-sepharose (GE Healthcare). Biotinylated peptides and associated binding partners were purified using streptavidin-sepharose beads (GE Healthcare, Chicago, IL, USA) under gentle rotation at 4°C for 2 h. For immunopurification of PLCγ2, 2 μg of rabbit anti-PLCγ2 antibodies (#Q-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the lysate of 5 × 10^7 cells followed by incubation on a rotator for 4 h at 4°C. Subsequently, 25 μL protein A/G PLUS agarose beads (Santa Cruz Biotechnology) were added, followed by additional incubation for 1 h on a rotator at 4°C. All affinity-purification samples were repeatedly washed in lysis buffer, before they were subjected to SDS-PAGE and immunoblotting. Antibodies for immunopurification and immunoblotting: the monoclonal antibodies against β-Actin (clone 13E5), SLP65/BLNK (clone D8R3G), Vav1 (clone D45G3), phospho-p38 MAPK (Thr180/Tyr182, clone 3D7), phosphotyrosine (clone 100) and the polyclonal anti-p38 MAP kinase antibody, were from Cell Signaling Technology (Danvers, MA, USA). The monoclonal antibodies to Vav2 (clone EP1067Y), CD79A (Igκ, clone EP3618), and to CD79B (Igκ, clone EPR6861) were from Abcam (Cambridge, UK). The polyclonal anti-PLCγ2 antibody (Q-20), anti-Rac2 (C-11), and the monoclonal anti-Syk antibody (clone 4D10) were from Santa Cruz Biotechnology. Rabbit polyclonal anti-Vav3 was from Merck Millipore (Burlington, MA, USA) and the polyclonal anti-GST antibody was from Molecular Probes (Eugene, OR, USA). Monoclonal anti-Rac1 (ARC03) was from Cytoskeleton Inc. (Denver, CO, USA). Biotinylated (phospho-) peptides encompassing the intracellular regions of Igκ or Igβ were synthesized by CASLO ApS (Lyngby, Denmark) or by Eurogentec (Li`ege, Belgium). Immunoblot images were processed using Photoshop CS4 and Corel Draw software. Quantification of band intensities was done with LabImage 1D software (Kapel Bio-Imaging, Leipzig, Germany). The inhibitors EHop-016, Rhosin, and Latrunculin A were from Calbiochem (Merck Millipore).

Measurement of intracellular free Ca^{2+}

The day before the measurement, ~1 x 10^6 cells were seeded in a 10-cm culture dish. The next day, cells were loaded under gentle mixing for 30 min at 30°C with 1 μM Indo-1-AM (Invitrogen, Thermo Fisher Scientific) in RPMI containing 10% fetal bovine serum and 0.015% Pluronics-F-127 (Invitrogen). Subsequently, cells were washed twice and resuspended in Kreb’s-Ringer solution composed of 10 mM HEPES (pH 7.0), 140 mM NaCl, 10 mM glucose, 4 mM KCl, 1 mM MgCl2, and 1 mM CaCl2. After 30 min of resting prior to the measurement, the fluorescence ratio of Ca^{2+}-bound Indo-1 (405 nm) to Ca^{2+} unbound Indo-1 (530 nm) was monitored on an LSR II cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The basal Indo-1-AM ratio was monitored for 25 s, followed by stimulation with either 20 μg/mL goat anti-human IgM F(ab')2 (Jackson ImmunoResearch) or 10 μg/mL anti-human CD8 (clone MEM-31, a gift of Dr. Vaclav Horejsi, Prague, Czech Republic). Data were analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA), Microsoft Excel, and GraphPad Prism. A forward scatter/side scatter gate as shown in Supporting information Fig. S11 was applied in each analysis to exclude cellular debris.

Intracellular flow cytometry

For analysis of phospho-Akt or phospho-SLP65, roughly 1.5 x 10^6 cells were seeded in a 10-cm culture dish the day before the experiment. Prior to stimulation with 20 μg/mL goat anti-human IgM F(ab')2 (Jackson ImmunoResearch, Cambridge, UK), cells were rested for 30 min in FBS-free RPMI at 37°C. After stimulation, cells were fixed with CytoFix (1:1, BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at 37°C and subsequently permealized with Perm/Wash Buffer I (BD Biosciences) for 20
min at 22°C. Afterwards, cells were stained with anti-phospho-Akt (pS473) Alexa Fluor 647 (Cell Signaling Technology #4075, dilution 1:100) or anti-phospho-SLP65-AF647 (pY84, clone J117-1278, BD Biosciences, dilution 1:6) for 45 min at 22°C. After washing the cells with Perm/Wash Buffer I, the fluorescence was measured using an LSR II cytometer (Becton Dickinson). Data analysis was done with FlowJo (FlowJo LLC), Microsoft Excel and GraphPad Prism. For measurements of actin polymerization, ~2.5 × 10⁶ cells were seeded in a 10-cm culture dish 24 h before the experiment. Twenty minutes before BCR stimulation, 0.5 × 10⁶ cells of a citrine-negative and -positive cell line were mixed in a 1:1 ratio and rested at 37°C in FBS-free RPMI. After stimulation with 20 μg/mL goat antihuman IgM F(ab′)² for the indicated times, cells were fixed with CytoFix (1:1, BD Biosciences), permealized with 50 μg/mL lysophosphatidylcholine (Sigma-Aldrich) and stained with Phalloidin-AlexaFluor 647 (Thermo Fisher Scientific, dilution 1:40) for 20 min at 37°C under mild agitation. Afterwards, cells were washed with PBS containing 2% BSA followed by flow cytometric analysis using an LSR II cytometer (Becton Dickinson). Data were analyzed with FlowJo, Microsoft Excel, and GraphPad Prism.

GEF activity assay

The constructs of Vav family members used for the guanine nucleotide exchange assay were expressed with an N-terminal His₁₀-tag in E.coli BL21-CodonPlus cells. Proteins were purified with HisPur Ni-NTA Superflow Agarose (Thermo Fisher Scientific). Guanine nucleotide exchange assays were carried out using Bodipy-GDP-bound small G proteins was measured every 30 s for 20 min at 20°C. Bodipy-GDP-bound small G proteins were added. The fluorescence of purified Vav protein fragments were added. The fluorescence of Bodipy-GDP-bound small G proteins was measured every 30 s for 20 min at 20°C using a Cytation3-Imager (BioTek Instruments, Inc, Winooski, VT, USA). The used Vav protein fragments were as follows: human Vav1: amino acids 170–575 carrying a Y174D substitution, human Vav1-DAR: 189–575, human Vav3: 169–573 (Y173D), murine Vav1: 170–575 (Y174D). Additional mutations are specified in the respective figure legends.

Isolation of primary human B cells

Primary human B cells from peripheral blood of healthy donors were isolated by magnetic cell sorting using the B-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The purity of the isolated B cells was tested by surface staining of CD19 (anti-CD19-PE clone LT19, Miltenyi Biotec) and IgM (anti-human IgM-AF647, Southern-Biotech, Birmingham, AL, USA), and was typically around 90% or higher. Experiments involving human participants were approved by the ethical review committee of the University Medical Center Göttingen and were performed in accordance with relevant guidelines and regulations. An informed consent was obtained from all participants.

Statistical analysis

Two-way ANOVA statistical analyses followed by Tukey’s or Sidak’s multiple comparison tests were done with GraphPad Prism software. Differences between groups were considered significant for p < 0.05, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Error bars in graphs show SD of mean values.

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Data availability statement: The datasets generated and analyzed during the current study as well as reagents and cell lines that were generated and used are available from the corresponding author on reasonable request.

Author Contributions: JL and CH designed and performed experiments and analyzed data. MH performed experiments. NE designed the research, made experiments, analyzed and interpreted data and wrote the paper.

Conflict of interest: The authors declare that they have no commercial or financial conflict of interest.

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Abbreviations: AR: acidic region · Btk: Bruton’s tyrosine kinase · DAG: diacylglycerol · DH: Dbl homology · GEFs: guanine nucleotide exchange factors · IP3: inositol-1,4,5-trisphosphate · LatA: Latrunculin A · PLCγ2: phospholipase Cγ2 · SH2: Src homology 2 · PTKs: protein tyrosine kinases · ZF: zinc finger

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