


PIP5K1 γ 90-generated phosphatidylinositol-4,5-bisphosphate promotes the uptake of *Staphylococcus aureus* by host cells

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Abstract

Staphylococcus aureus, a Gram-positive pathogen, invades cells mainly in an integrin-dependent manner. As the activity or conformation of several integrin-associated proteins can be regulated by phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂), we investigated the roles of PI-4,5-P₂ and PI-4,5-P₂-producing enzymes in cellular invasion by *S. aureus*. PI-4,5-P₂ accumulated upon contact of *S. aureus* with the host cell, and targeting of an active PI-4,5-P₂ phosphatase to the plasma membrane reduced bacterial invasion. Knockdown of individual phosphatidylinositol-4-phosphate 5-kinases revealed that phosphatidylinositol-4-phosphate 5-kinase γ (PIP5K1 γ) plays an important role in bacterial internalization. Specific ablation of the talin and FAK-binding motif in PIP5K1 γ 90 reduced bacterial invasion, which could be rescued by reexpression of an active, but not inactive PIP5K1 γ 90. Furthermore, PIP5K1 γ 90-deficient cells showed normal basal PI-4,5-P₂ levels in the plasma membrane but reduced the accumulation of PI-4,5-P₂ and talin at sites of *S. aureus* attachment and overall lower levels of FAK phosphorylation. These results highlight the importance of local synthesis of PI-4,5-P₂ by a focal adhesion-associated lipid kinase for integrin-mediated internalization of *S. aureus*.

KEYWORDS

fibronectin-binding protein, internalization, phosphatidylinositol-4,5-bisphosphate, phosphatidylinositol-4-phosphate-5-kinase, *Staphylococcus aureus*

1 | INTRODUCTION

Due to the rise in multidrug resistance, *Staphylococcus aureus*, a Gram-positive coccoid bacterium, is becoming a major healthcare challenge on a global scale (Liu et al., 2011; Sieradzki et al., 1999; Tsiodras et al., 2001; Turner et al., 2019). This microbial pathogen is able to cause a spectrum of diseases, from chronic manifestations such as abscesses, chronic osteomyelitis, or mastitis to life-threatening acute diseases such as endocarditis, sepsis, or toxic

shock syndrome. *S. aureus* produces numerous bacterial surface-associated and secreted virulence factors, which allow the pathogen to adhere to and invade otherwise nonphagocytic cells, to evade immune detection, or to intoxicate host tissues (Arciola et al., 2018; Foster et al., 2014). Among staphylococcal adhesins, the cell wall-anchored fibronectin-binding proteins (FnBPs) play a particularly prominent role in host cell invasion (Foster, 2016; Hauck et al., 2012; Sinha et al., 1999). FnBPs can capture the glycoprotein fibronectin (Fn), which is an abundant constituent of human blood plasma

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(Vuento & Vaheri, 1979). Soluble Fn adopts a closed, inactive conformation, where binding sites for receptors of the integrin family are inaccessible due to long-range intramolecular interactions between amino-terminal type I modules (FnI₁₋₃) and carboxy-terminal type III modules (FnIII₁₋₃ and FnIII₁₂₋₁₄) within the Fn homodimer (Henderson et al., 2011; Hymes & Klaenhammer, 2016; Vakonakis et al., 2009). Upon association of staphylococcal FnBP with Fn type I modules, intramolecular constraints are released exposing the integrin-binding motifs located in FnIII₉ and FnIII₁₀ (Marjenberg et al., 2010). As a consequence, FnBP-bound fibronectin is able to bind with high affinity to host receptors of the integrin family, such as integrin $\alpha_5\beta_1$, thereby tightly linking the bacteria with the host cell surface (Agerer et al., 2003; Sinha et al., 1999). FnBP-mediated Fn coating of the microorganisms clusters integrins, which in turn trigger canonical signaling events involving focal adhesion kinase (FAK) and Src family protein tyrosine kinases (PTKs) (Agerer et al., 2003, 2005; Fowler et al., 2003) and initiate the uptake of the bacteria by nonprofessional phagocytes such as fibroblasts, epithelial cells, and endothelial cells (Agerer et al., 2005; Brouillette et al., 2003; Dziewanowska et al., 1999; Konkel et al., 2020; Schroder et al., 2006).

Under physiological conditions, Fn-bound integrins initiate stable, localized multimeric protein complexes, so-called focal adhesions, which indirectly connect the intracellular domains of integrin heterodimers with the actomyosin cytoskeleton (Huvneers et al., 2008; Strohmeyer et al., 2017). Some of the structural integrin- or actin-associated proteins as well as F-actin are also recruited to Fn-coated *S. aureus* and are involved in the internalization of the bacteria (Agerer et al., 2005; Schroder et al., 2006). However, the regulatory checkpoints, which allow the bacteria to convert integrin-associated protein complexes into an endocytotic machinery, are currently unknown.

Interestingly, several of the integrin-associated proteins are regulated by phosphoinositides. In particular, phosphatidylinositol (PtdIns)-4,5-bisphosphate (PI-4,5-P₂) does not only accumulate and help orchestrate the plasma membrane association of integrin-linked structural and signaling proteins such as talin, α -actinin, vinculin, or FAK (Chinthalapudi et al., 2018; Goni et al., 2014; Janmey, 1994; Martin, 1998) but also plays key roles in endocytosis and membrane trafficking (Daste et al., 2017; Mandal, 2020; Oppelt et al., 2013; Posor et al., 2015). In mammalian cells, PI-4,5-P₂ is mainly generated at the inner leaflet of the plasma membrane by the activity of type I PI-4-P 5-kinases (PIP5Ks) (van den Bout & Divecha, 2009). The three known members of the PIP5K family, PIP5K α , PIP5K β , and PIP5K γ (Jenkins et al., 1994; Kwiatkowska, 2010), preferentially phosphorylate the 5-hydroxyl position of PI-4-P to generate PI-4,5-P₂. PIP5K α is enriched at the plasma membrane and the Golgi complex and has also been observed at sites of membrane ruffling induced by Rho GTPases. PIP5K β localizes to the plasma membrane and to vesicles in the perinuclear region of the cell (Di Paolo & De Camilli, 2006; Doughman et al., 2003). PIP5K γ is present at the plasma membrane, with at least one isoform, the so-called PIP5K γ 90 or PIP5K γ isoform #2, found to be enriched at focal adhesions and adherens junctions (Durand et al., 2016; Ling et al., 2002; Nader et al., 2016).

Take aways

- Fibronectin-binding *Staphylococcus aureus* induce accumulation of phosphoinositides at sites of host cell contact.
- Accumulation of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) is linked to *S. aureus* internalization.
- The integrin-associated enzyme PIP5K γ 90 is instrumental for local PI-4,5-P₂ production.
- Lack of PIP5K γ 90 impairs *S. aureus*-mediated integrin signaling and bacterial uptake.

With regard to bacterial internalization, PI-4,5-P₂ has been reported to either promote or inhibit the uptake of several bacteria. For example, PIP5K α and PI-4,5-P₂ synthesis are instrumental for the cellular entry of *Chlamydia caviae* into HeLa cells (Dautry-Varsat et al., 2005). A positive role of PIP5K α has also been reported for the internalization of *Yersinia pseudotuberculosis* (Wong & Isberg, 2003). In contrast, the Gram-negative pathogen *Shigella flexneri* injects the phosphoinositide phosphatase IpgD into the host cell, which dephosphorylates PI-4,5-P₂ and facilitates bacterial uptake (Niebuhr et al., 2002). Similarly, entry of *Salmonella typhimurium* and *Y. pseudotuberculosis* into host cells requires dephosphorylation of PI-4,5-P₂ to promote plasma membrane scission of the forming bacteria-containing vacuoles indicating that PI-4,5-P₂ hinders efficient internalization in these instances (Sarantis et al., 2012; Terebiznik et al., 2002).

In the case of *S. aureus*, however, it is unknown whether PI-4,5-P₂ is generated and whether it positively or negatively contributes to the integrin-mediated uptake of bacteria. We investigated this question in the present study and report that high levels of PI-4,5-P₂ occur at sites, where *S. aureus* engages with integrins. *S. aureus* internalization is reduced upon overexpression of a membrane-targeted PI-4,5-P₂-directed phosphatase or upon reduction of PIP5K activity. Moreover, deletion of the gene encoding the 90 kDa isoform of PIP5K γ , which accumulates at integrin-rich sites associated with *S. aureus*, reduces both local PI-4,5-P₂ accumulation as well as bacterial internalization. Our results identify PI-4,5-P₂ as a positive contributor to host cell invasion by *S. aureus* and suggest that the lipid kinase PIP5K γ serves as a regulator of integrin internalization.

2 | RESULTS

2.1 | PI-4,5-P₂ and PI-3,4,5-P₃ are enriched at the attachment sites of *S. aureus*

Previous studies have revealed that *S. aureus* triggers the recruitment and activation of integrin-associated signaling molecules (Hauck et al., 2012). To investigate, if staphylococcal engagement

of integrins is accompanied by elevated levels of phosphoinositides, we transfected 293 cells with vectors encoding GFP-tagged PH domains or with a GFP-only encoding vector (Balla, 2005; Balla & Varnai, 2009). The selected PH domains recognizing distinct phosphoinositides were expressed at comparable levels (Figure S1a,b, Supplementary Material). Cells were infected with the *S. aureus* strain Cowan, which exploits the FnBP-generated Fn coat to engage host integrin $\alpha_5\beta_1$. Upon *S. aureus* binding, GFP distribution in the cytosol remained unaltered (Figure 1). In contrast, the PH domains of Akt and Btk, which recognize phosphatidylinositol-3,4,5 trisphosphate (PI-3,4,5-P₃) and which were found at low levels at the cell periphery of uninfected cells, were strongly recruited to cell-associated *S. aureus* 2 hr after infection (Figure 1). Furthermore, the PH domain of PLC δ , which specifically binds phosphatidylinositol-4,5 bisphosphate (PI-4,5-P₂), became highly enriched around cell-associated bacteria (Figure 1). In contrast, the phosphatidylinositol-4 phosphate (PI-4-P) and Arf-1-binding PH domain of OSBP, which directs this protein to the trans-Golgi-network (Levine & Munro, 2002), was not altered in its distribution upon *S. aureus* infection (Figure 1). These results indicated that PI-4,5-P₂ and PI-3,4,5-P₃ accumulate at *S.*

aureus-host cell attachment sites. As PI-4,5-P₂ is involved in regulating integrin-associated focal adhesion proteins, we concentrated on this phosphoinositide.

2.2 | PI-4,5-P₂ enrichment at the attachment sites of *S. aureus* is not a consequence of increased membrane volume

To confirm that the observed enrichment of PI-4,5-P₂ is not due to an increased overall volume of plasma membrane around cell-associated bacteria, we coexpressed a membrane-anchored red fluorescent protein (mKate-CAAX) together with either PLC δ -PH-GFP or GFP and infected the cells for 2 hr with pacific-blue stained *S. aureus* (Figure 2a). While the PLC δ -PH-GFP signal was enriched around *S. aureus*, mKate-CAAX showed a patchy distribution in the plasma membrane but was not enriched around cell-associated bacteria (Figure 2a). To better delineate the bulk plasma membrane, cells expressing GFP or PLC δ -PH-GFP were additionally stained with the membrane dye Cellmask Orange, which uniformly labeled the

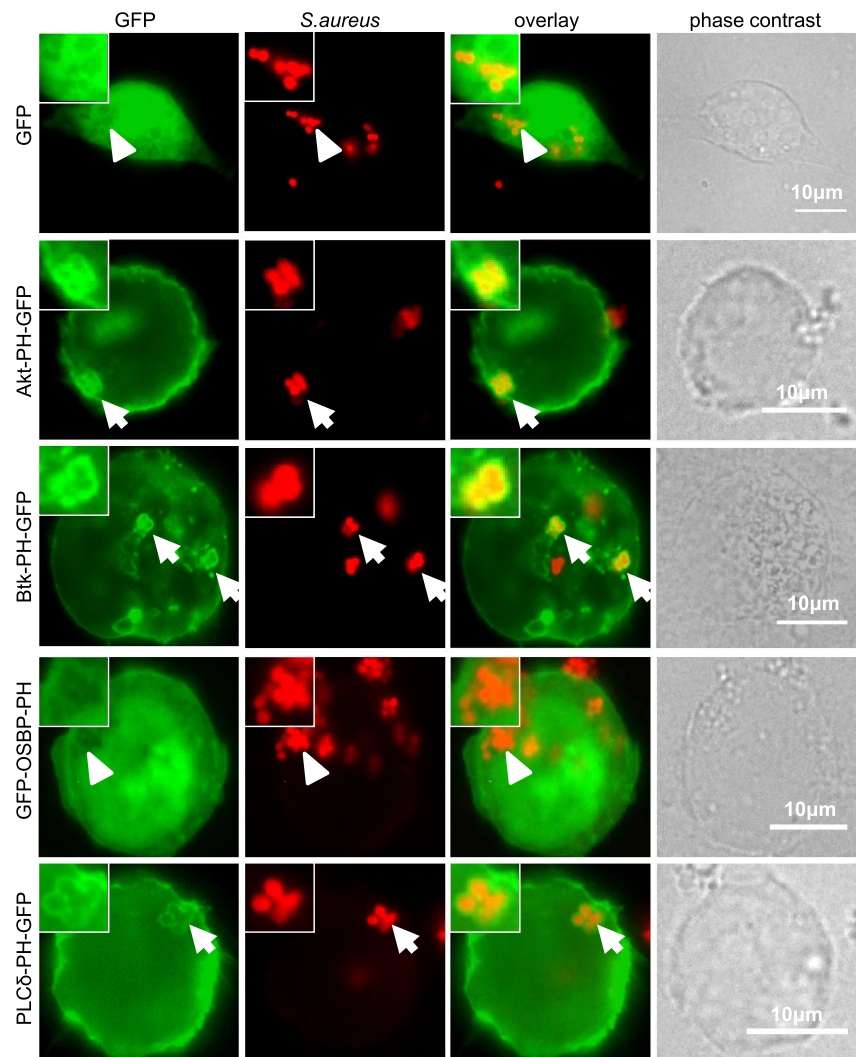


FIGURE 1 PI-4,5-P₂ and PI-3,4,5-P₃ are recruited to the attachment sites of *Staphylococcus aureus*. 293 cells were transfected with constructs encoding GFP or the indicated PH domains fused to GFP. Twenty-four hours after transfection, cells were seeded on poly-L-lysine-coated coverslips and infected with rhodamine-labeled *S. aureus* for 2 hr before fixation. The unaltered distribution of GFP, as well as OSBP-PH-GFP (arrowheads) and recruitment of various PH-domain-GFP-fusion proteins to cell-associated bacteria (arrows), was visualized by widefield fluorescence microscopy. Bar, 10 μ m

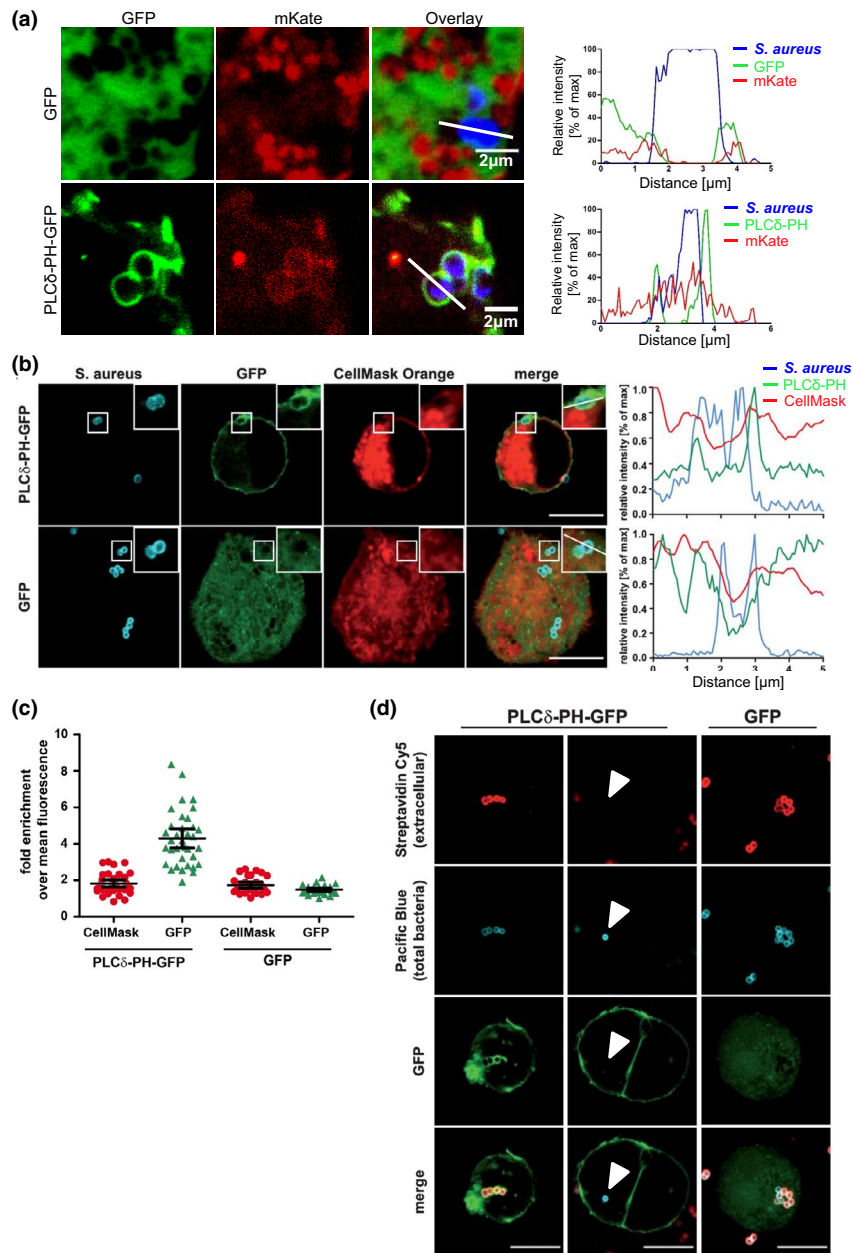


FIGURE 2 PI-4,5-P₂ accumulates around cell-associated *Staphylococcus aureus*. (a) 293 cells were cotransfected with pcDNA3.1-mKate-CAAX together with GFP or PLC δ -PH-GFP. Twenty-four hours after transfection, cells were seeded on poly-lysine-coated coverslips and infected with pacific blue-labeled *S. aureus* for 2 hr. Using confocal microscopy, the recruitment of mKate-CAAX, GFP, or PLC δ -PH-GFP to cell-associated bacteria (blue) was monitored. Fluorescence intensity profiles (right graphs) along with the line indicated in the overlay picture show the strong enrichment of PLC δ -PH-GFP versus mKate-CAAX around *S. aureus* (blue). (b) 293 cells were transfected with PLC δ -PH-GFP or GFP, infected with pacific blue-labeled *S. aureus*, and stained with a membrane dye (CellMask Orange). Fluorescence intensity profiles along with the line show that the PLC δ -PH-GFP recruitment is not due to membrane volume effects. Scale bars, 10 μ m. (c) Quantification of the experiment in (b). 25–35 individual infection sites were analyzed by measuring the maximum intensity of the CellMask Orange stain or GFP at the site and dividing it by the mean fluorescence intensity of the whole cell. The fold enrichment over the mean fluorescence of the respective fluorophores is given. Error bars indicate 95% confidence intervals. (d) *S. aureus* were labeled with pacific blue and Sulfo-NHS-Biotin and used for infection of PLC δ -PH-GFP or GFP transfected 293 cells. Following infection, extracellular bacteria were additionally labeled with Streptavidin-Cy5. Shown are representative PLC δ -PH-GFP expressing cells with cell-associated *S. aureus* (left row) or internalized *S. aureus* (middle row; arrowhead). No recruitment of GFP was seen (right row). Scale bars, 10 μ m

plasma membrane (Figure 2b). Again, PLC δ -PH-GFP, but not GFP, showed a clear enrichment around *S. aureus*-host cell contact sites (Figure 2b). Quantification of the relative fluorescence intensities

at these contact sites versus other areas of the cell membrane revealed that PH-PLC δ -GFP was ~5-fold enriched around the pathogens, whereas the general membrane stain showed only a minor

increase (~1.9-fold) in fluorescence around cell-associated bacteria (Figure 2c). These data indicate that the membrane volume around cell-attached bacteria slightly increases, as expected for an endocytic process, but that there is a much more prominent elevation in PI-4,5-P₂ levels at these sites (Figure 2c). Moreover, the accumulation of PI-4,5-P₂ around the bacteria occurred upon contact with the host cell, when the microbes were still extracellular, whereas the PLCδ-PH-GFP signal around the bacteria was lost upon internalization (Figure 2d). Together, these data demonstrate that PI-4,5-P₂ accumulates at *S. aureus*-host cell attachment sites.

2.3 | PI-4,5-P₂ accumulation is induced by FnBP-mediated integrin engagement

In contrast to nonpathogenic members of the genus *Staphylococcus*, *S. aureus* can recruit Fn and thereby engage integrins on the cell surface. To test whether the observed accumulation of the PI-4,5-P₂-binding PLCδ-PH-GFP is due to integrin engagement, cells were infected with *S. aureus* or the nonpathogenic, non-Fn-binding *S. carnosus*, respectively. Whereas infection with *S. aureus* resulted in a strong accumulation of PLCδ-PH-GFP, the rare contact sites

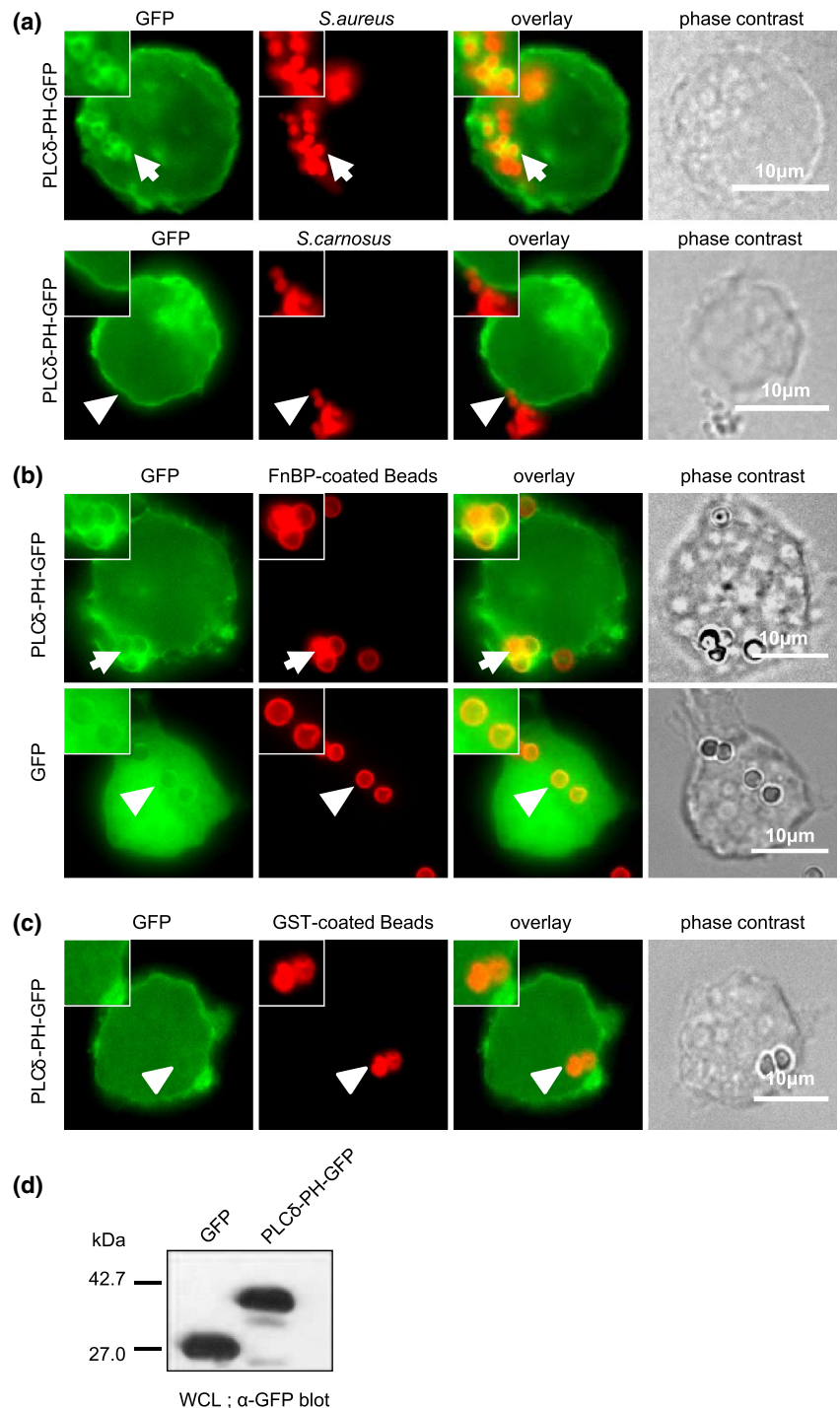


FIGURE 3 PI-4,5-P₂ accumulation is induced by FnBP-mediated integrin engagement. (a) 293 cells were transfected with PLCδ-PH-GFP, then infected by rhodamine stained *Staphylococcus aureus* or *S. carnosus* for 2 hr, respectively. Infected samples were fixed and observed by widefield microscopy. Recruitment of PLCδ-PH-GFP is seen around cell-associated *S. aureus* (arrows) but not *S. carnosus* (arrowheads). (b, c) 293 cells were transfected with vectors encoding PLCδ-PH-GFP or GFP, respectively. Transfected cells were incubated with FnBP-coated (b) or GST-coated (c) microbeads for 2 hr. After incubation, cells were fixed and analyzed as in (a). PLCδ-PH-GFP (arrows), but not GFP (arrowheads), was recruited to GST-FnBP-coated beads (b), whereas GST-coupled beads did not lead to recruitment of PLCδ-PH-GFP (c). Bar, 10 μm. (d) Expression of GFP and PLCδ-PH-GFP by transfected 293 cells was detected by Western Blotting with a monoclonal anti-GFP antibody

of *S. carnosus* with host cells did not show recruitment of PLC δ -PH-GFP (Figure 3a). To test whether bacterial binding via FnBP to integrins is sufficient to induce increased PI-4,5-P₂ levels around cell-associated bacteria, the fibronectin-binding domains of FnBP were expressed as GST-fusion protein in *Escherichia coli*, purified, covalently attached to microbeads and then added to 293 cells expressing the GFP-tagged PLC δ PH domain or GFP only. Similar to the intact, Fn-binding *S. aureus*, GST-FnBP-coated beads triggered PLC δ PH-GFP recruitment, whereas GFP distribution within the cell remained unaltered (Figure 3b). As expected, microbeads coated with GST associated poorly with cells and did not result in accumulation of PLC δ -PH-GFP (Figure 3c). Equivalent expression levels of PLC δ PH-GFP or GFP were verified by Western blotting (Figure 3d). Altogether, these findings demonstrate that FnBP-mediated host cell contact by *S. aureus* is sufficient to trigger a local elevation in PI-4,5-P₂ levels.

2.4 | Reduction of plasma membrane PI-4,5-P₂ inhibits *S. aureus* invasion

To investigate if the elevated PI-4,5-P₂ levels observed in the vicinity of cell-associated *S. aureus* are relevant for the internalization of the bacteria, we attempted to directly interfere with the plasma membrane pools of PI-4,5-P₂ by overexpressing a PI-4,5-P₂ consuming enzyme. To this end, we fused the myristoylation sequence derived from the Src family kinase Lyn and GFP to the intact phosphatase domain of the 5'-inositol phosphatase INPP5J (myrINPP5J-GFP) or to a phosphatase-inactive INPP5J (myrINPP5J-ia-GFP). The resulting proteins were expressed at equivalent levels by 293 cells (Figure S2a). The transfected cells were infected with *S. aureus* for 2 hr and differentially stained for intra- and extracellular bacteria (Figure S2b) to quantify the total cell-associated and the internalized bacteria. While the total number of cell-associated bacteria was similar in the different samples, expression of active myrINPP5J slightly, but consistently reduced the internalization of *S. aureus* (Figure 4a,b). To investigate if the active myrINPP5J affects PI-4,5-P₂ levels at the bacterial attachment sites, cells were cotransfected with either myrINPP5J or myrINPP5J-ia fused to the blue fluorescent protein Cerulean together with PLC δ PH-GFP. PI-4,5-P₂ accumulation around *S. aureus* was less pronounced in myrINPP5J expressing cells compared with cells expressing myrINPP5J-ia or the isolated Cerulean, while the total cellular levels of PI-4,5-P₂ were only marginally reduced (Figure 4c). The overexpression of myrINPP5J lowered PLC δ PH-GFP accumulation by about 40% (Figure 4d). This partial effect might be due to the predominant localization of myrINPP5J to internal membranes (Figure S2b), rather than to the plasma membrane, where *S. aureus* internalization is taking place. Nevertheless, the results with myrINPP5J overexpression indicated that the increased levels of PI-4,5-P₂ found around cell-associated *S. aureus* contribute to bacterial internalization.

2.5 | PIP5K1 γ promotes the uptake of *S. aureus*

PI-4,5-P₂ can be generated by the action of PIP kinases or by dephosphorylation of PI-3,4,5-P₃. The major contributors to the generation of plasma membrane PI-4,5-P₂ pools comprise the enzymes of the type I phosphatidylinositol 4-phosphate 5-kinase (PIP5KI) family (Mayering, 2012). In humans, the PIP5KI family consists of three isozymes, α , β , and γ (Ishihara et al., 1996, 1998; Loijens & Anderson, 1996). To investigate, which of these three enzymes contributes to *S. aureus* host cell invasion, we efficiently depleted the different PIP5KIs mRNAs in HeLa cells using specific siRNAs (Figure 5a), infected the resulting cells with *S. aureus* or *S. carnosus*, and determined the number of total cell-associated as well as of viable intracellular bacteria (Figure 5b). Interestingly, the amount of total cell-associated bacteria between control cells and the different knock-down cells was comparable (Figure 5b), suggesting that the surface expression and ligand binding ability of integrin $\alpha_5\beta_1$ was not affected by the depletion of either enzyme. While the number of viable intracellular bacteria was unaltered in PIP5KI β -knockdown cells, the recovered intracellular bacteria were significantly decreased in PIP5KI γ - and PIP5KI α -knockdown cells (Figure 5b). As PIP5KI γ is known to interact with proteins at integrin-based focal adhesion sites, we focused our attention on this enzyme. First, we investigated the subcellular location of PIP5KI γ during bacterial uptake by expressing a GFP-tagged wild type or a kinase-inactive form of PIP5KI γ in 293 cells (Figure S3). Both the wild type and the inactive form of PIP5KI γ localized to the plasma membrane, while, in particular, the wildtype enzyme showed a prominent, more than 10-fold enrichment around the *S. aureus* attachment sites (Figure 5c,e). Clearly, an accumulation of PIP5KI γ was not observed upon infection with *S. carnosus* (Figure 5d). Collectively, these data indicate that PIP5KI γ localizes to sites of *S. aureus* internalization and support the idea that this enzyme provides local PI-4,5-P₂ production.

2.6 | Deletion of the PIP5KI γ talin-binding site impairs uptake of *S. aureus*

In mammalian cells, there are two main isoforms of PIP5KI γ , which are named, according to their molecular size, PIP5KI γ 90 or PIP5KI γ 87 (Figure 6a). Due to alternative splicing, the PIP5KI γ 87-encoding transcript skips exon 17 and therefore lacks a 26 amino acid motif near the carboxy-terminus (van den Bout & Divecha, 2009). This short amino acid stretch binds to the integrin-associated protein talin and is important to localize PIP5KI γ 90 to integrin-rich focal adhesion sites (Di Paolo et al., 2002). There, PIP5KI γ 90 is involved in a local production of PI-4,5-P₂ (Ling et al., 2002). Interestingly, mice with a genetic deletion of exon 17 of the *pip5kl γ* gene (PIP5KI γ 90^{-/-}) are viable and fertile and express PIP5KI γ 87 but lack the PIP5KI γ 90 isoform (Legate et al., 2012). To directly test the role of PIP5KI γ 90 and its 26aa talin-binding motif, we established murine embryonic fibroblasts (MEFs) derived from mice carrying a floxed exon 17 of the *pip5kl γ* gene (PIP5KI γ 90^{fl/fl} control cells) or lacking exon 17

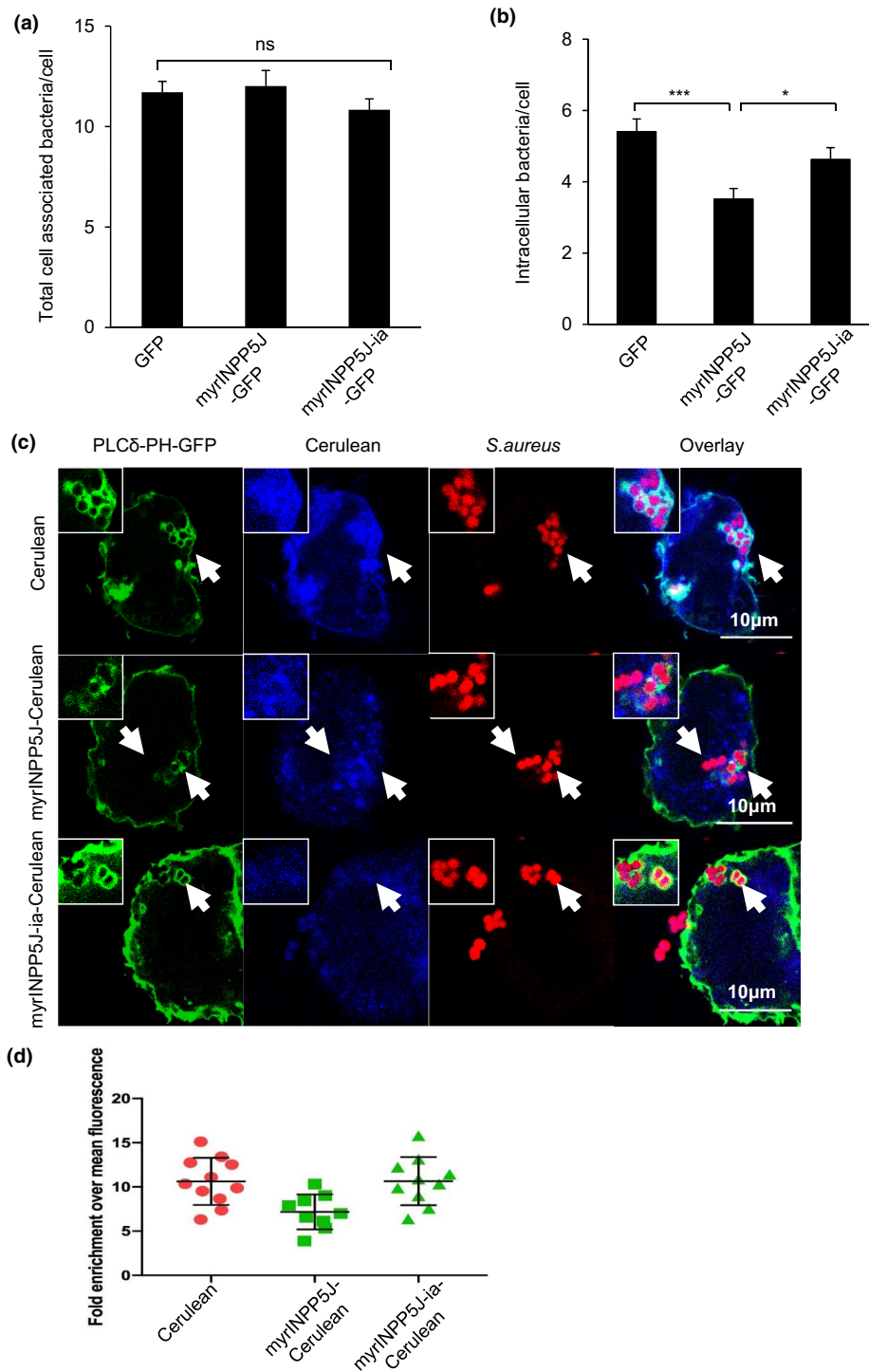


FIGURE 4 Expression of a membrane-targeted 5'-PIP phosphatase domain decreases *Staphylococcus aureus* internalization. (a and b) 293 cells were transfected with GFP, the membrane-targeted phosphatase domain of INPP5J (myrINPP5J-GFP), or the inactive phosphatase (myrINPP5J-ia-GFP), respectively. Twenty-four hours after transfection, 2×10^5 cells were seeded on poly-L-lysine-coated coverslips and infected the next day with pacific blue-stained and biotin-labeled *S. aureus* for 2 hr. After fixation, samples were incubated with streptavidin-AlexaFluor647 to mark extracellular bacteria. Total cell-associated (a) or intracellular (b) bacteria were quantified in three independent samples ($n = 3$ samples; at least 70 cells/sample). Bars represent mean \pm SEM of bacteria/cell; unpaired t test. *** $p < .001$, * $p < .05$. (c) 293 cells were cotransfected with PLCδ-PH-GFP together with Cerulean, the membrane-targeted phosphatase domain of INPP5J (myrINPP5J-Cerulean), or the inactive phosphatase (myrINPP5J-ia-Cerulean), respectively. Twenty-four hours after transfection, 2×10^5 cells were seeded on poly-L-lysine-coated coverslips and the next day infected with rhodamine-stained *S. aureus* for 2 hr. Fixed samples were analyzed by confocal microscopy, arrows point to *S. aureus*-cell attachment sites, which show reduced recruitment of PLCδ-PH-GFP in cells expressing myrINPP5J-Cerulean. (d) PLCδ-PH-GFP enrichment at infection sites of cells from (c) was analyzed by measuring the maximum GFP intensity at the infection site and dividing it by the mean fluorescence intensity of the whole cell. The fold enrichment of 9–11 individual sites is shown in the scatter plot with bars indicating the mean fold enrichment and error bars indicate 95% confidence intervals

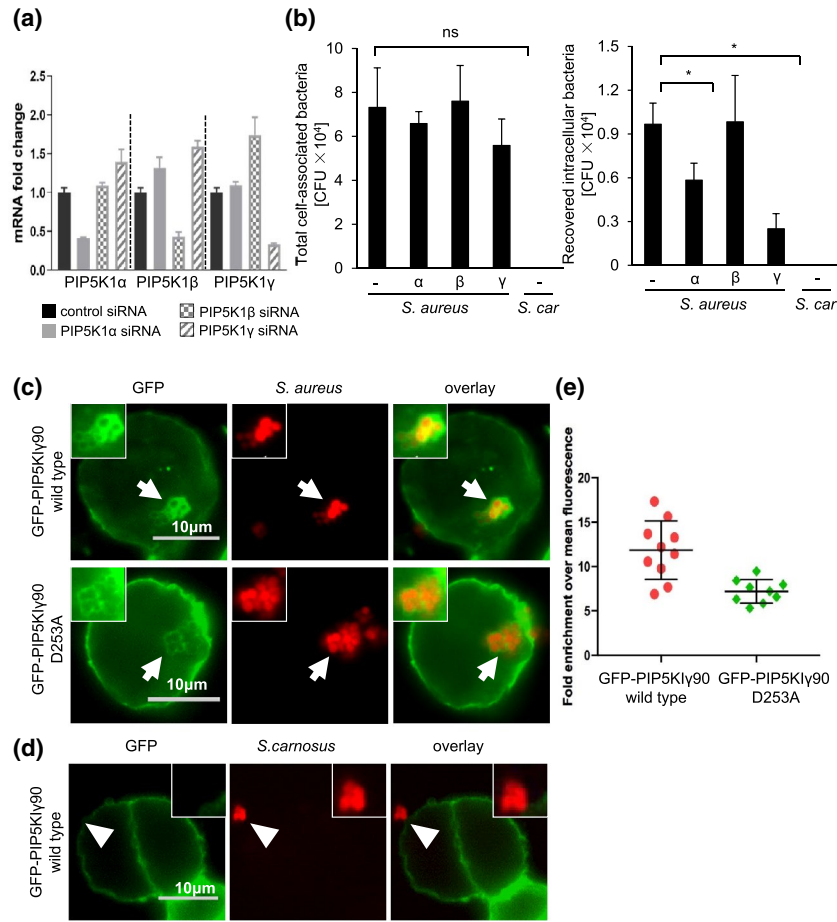


FIGURE 5 PIP5K1 γ is involved in the integrin-mediated uptake of *Staphylococcus aureus*. (a) HeLa cells were transfected with siRNA against PIP5K1 α , β , γ , or with control siRNA, respectively. Forty-eight hours later, total RNA was isolated and the mRNA level for each PIP5K1 transcript was analyzed by qRT-PCR. PIP5K1 transcription was normalized to GAPDH level and the Ct values from control knockdown samples are used to calculate the fold change of mRNA levels. Bars represent the mean \pm SEM of three independent experiments each performed in triplicate. (b) siRNA-transfected HeLa cells were infected for 2 hr with *S. aureus* or *S. carnosus*, respectively. Total cell-associated or intracellular bacteria were quantified by gentamicin protection assay. The bars show mean values \pm SEM of three independent experiments. Samples were compared with control knockdown cells infected with *S. aureus* and significance was evaluated by an unpaired *t* test. **p* < .05. (c, d) 293 cells were transfected as indicated with the GFP-tagged active (PIP5K1 γ 90 wild type) or the enzymatic inactive form of PIP5K1 γ (PIP5K1 γ 90 D253A). Transfected cells were infected with rhodamine-labeled *S. aureus* (c) or *S. carnosus* (d), respectively. Two hours later, the samples were fixed and analyzed by confocal microscopy. The recruitment of GFP-PIP5K1 γ 90 wild type and GFP-PIP5K1 γ 90 D253A in response to *S. aureus* infection is indicated by arrows (c), whereas *S. carnosus* does not lead to altered distribution of GFP-PIP5K1 γ 90 (d; arrowhead). (e) Local accumulation of GFP-PIP5K1 γ 90 wild type and GFP-PIP5K1 γ 90 D253A at infection sites of cells from (c) was analyzed by measuring the maximum GFP intensity at the infection site and dividing it by the mean fluorescence intensity of the whole cell. The fold enrichment of 9–10 individual sites is shown in the scatter plot with bars indicating the mean fold enrichment and error bars indicate 95% confidence intervals

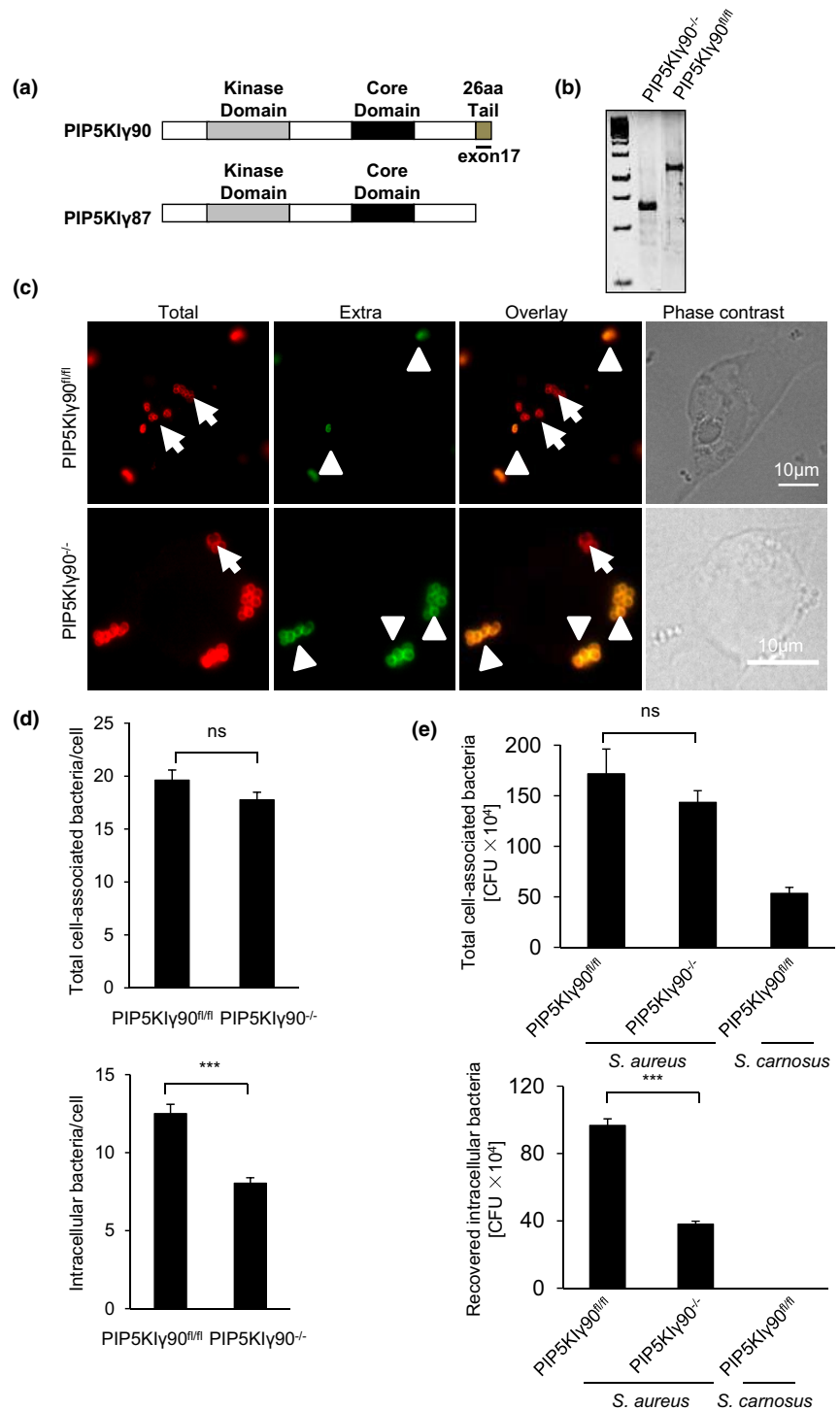
(PIP5K1 γ 90 $^{-/-}$). We confirmed the deletion of exon 17 by genotyping (Figure 6b) and verified similar expression levels of fibronectin-binding integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ as well as integrin-associated proteins by flow cytometry or Western blotting, respectively (Figure S4a,b). PIP5K1 γ 90 $^{fl/fl}$ control and PIP5K1 γ 90 $^{-/-}$ cells were infected with *S. aureus* for 2 hr, fixed, and differentially stained for intra- and extracellular bacteria (Figure 6c,d). Quantification of total cell-associated or intracellular bacteria in PIP5K1 γ 90 $^{fl/fl}$ versus PIP5K1 γ 90 $^{-/-}$ cells demonstrated that bacterial binding occurred at a similar level, but the number of intracellular bacteria in PIP5K1 γ 90 $^{-/-}$ cells was significantly lower than in PIP5K1 γ 90 $^{fl/fl}$ cells (Figure 6d). Similar results were obtained by gentamicin protection assays. Again, the levels of total cell-associated bacteria did not differ, while less viable

intracellular bacteria were recovered from PIP5K1 γ 90 $^{-/-}$ cells compared with PIP5K1 γ 90 $^{fl/fl}$ cells (Figure 6e). Together, these results demonstrate that PIP5K1 γ 90 is responsible for local production of PI-4,5-P $_2$ to allow optimal integrin-mediated uptake of *S. aureus*.

2.7 | Reexpression of PIP5K1 γ 90 in PIP5K1 γ 90 $^{-/-}$ fibroblasts rescues uptake of bacteria

To confirm the contribution of PIP5K1 γ 90 to integrin-mediated internalization of *S. aureus*, the PIP5K1 γ 90 $^{-/-}$ cells were complemented by transiently expressing comparable levels of GFP-tagged PIP5K1 γ 90, the inactive version of PIP5K1 γ 90 (PIP5K1 γ 90 D253A) or GFP-only

FIGURE 6 Deletion of the PIP5K talin-binding site in fibroblasts impairs uptake of *Staphylococcus aureus*. (a) Schematic view of the two major PIP5K γ isoforms, PIP5K γ 90 and PIP5K γ 87, and the location of exon 17. (b) Genomic DNA of PIP5K γ 90 $^{-/-}$ and PIP5K γ 90 $^{fl/fl}$ cells was analyzed by PCR for the absence of exon 17. (c) PIP5K γ 90 $^{-/-}$ and PIP5K γ 90 $^{fl/fl}$ cells were infected with *S. aureus* for 2 hr. Fixed samples were differentially stained to distinguish the extracellular (red and green staining, arrowheads) and intracellular bacteria (red staining only; arrows). Bars, 10 μ m. (d) Experiments were performed as in (c) and the number of total cell-associated (upper panel) or intracellular bacteria (lower panel) per cell was quantified (at least 100 cells/sample). Significance was evaluated by an unpaired *t* test. $***p < .001$. (e) PIP5K γ 90 $^{fl/fl}$ and PIP5K γ 90 $^{-/-}$ cells were infected with *S. aureus* or *S. carnosus*, respectively, for 2 hr. The total cell-associated and recovered intracellular bacteria were quantified by gentamicin protection assay. The bars show mean values \pm SEM of three independent experiments. Significance was evaluated by an unpaired *t* test. $***p < .001$



(Figure S4c). These cells were infected with *S. aureus*, fixed, and differentially stained for extra- and intracellular bacteria (Figure 7a). Enumeration of bacteria in these transfected cells by microscopy revealed that the number of cell-associated bacteria was similar in all the samples (Figure 7b). However, the internalization of *S. aureus* by PIP5K γ 90 $^{-/-}$ cells was clearly diminished compared with wild-type cells (Figure 7b). Importantly, the uptake of *S. aureus* was rescued in PIP5K γ 90 $^{-/-}$ cells reexpressing the wild-type PIP5K γ 90 enzyme, whereas reexpression of the kinase-inactive enzyme or expression of GFP-only did not increase the numbers of intracellular *S. aureus*

(Figure 7a,b). These results corroborate the functional role of PIP5K γ 90 and its enzyme activity in the integrin-mediated uptake of *S. aureus*.

2.8 | The talin-binding site of PIP5K γ promotes the enrichment of PI-4,5-P $_2$ and talin at bacterial attachment sites

Since PIP5K γ was recruited to the cell-attached bacteria, we wondered whether this enzyme is responsible for the elevated PI-4,5-P $_2$

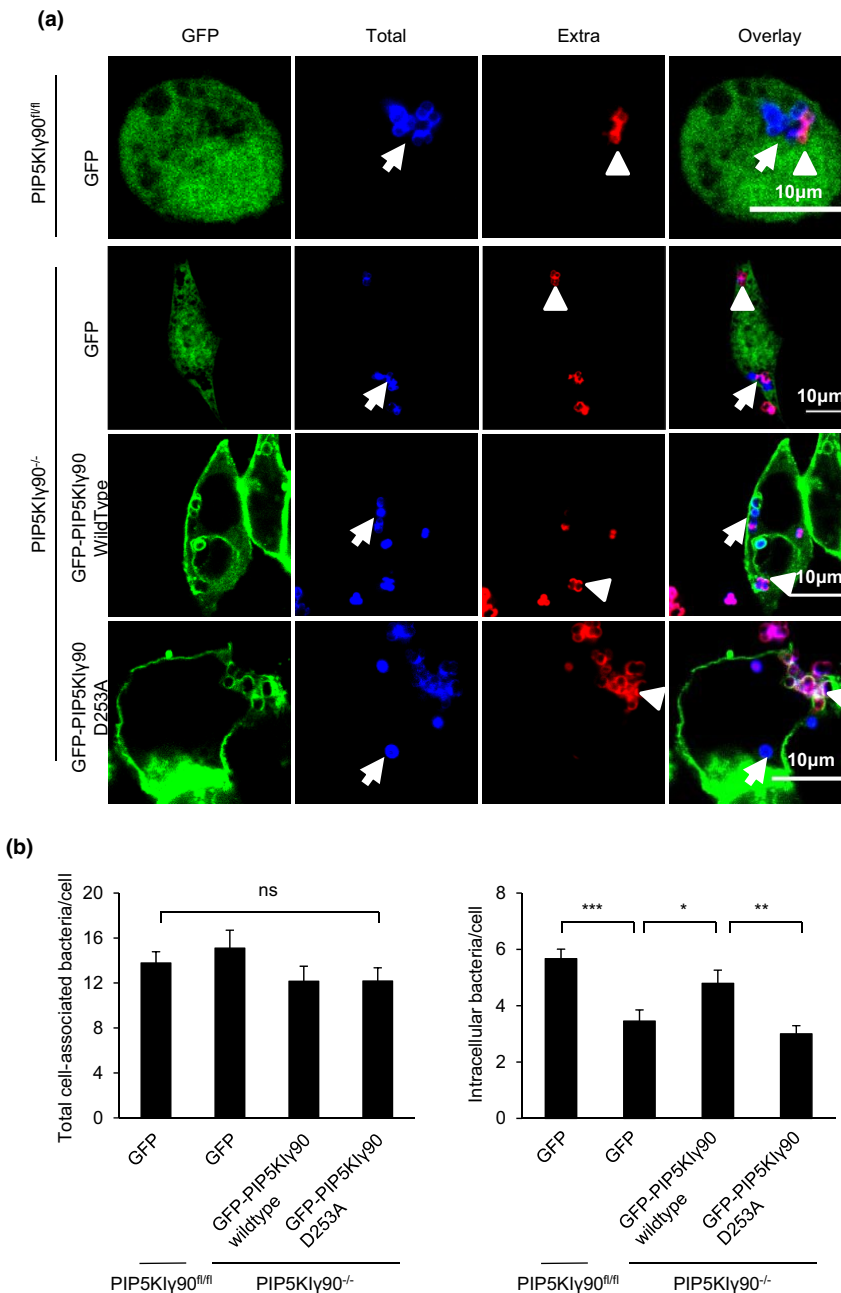
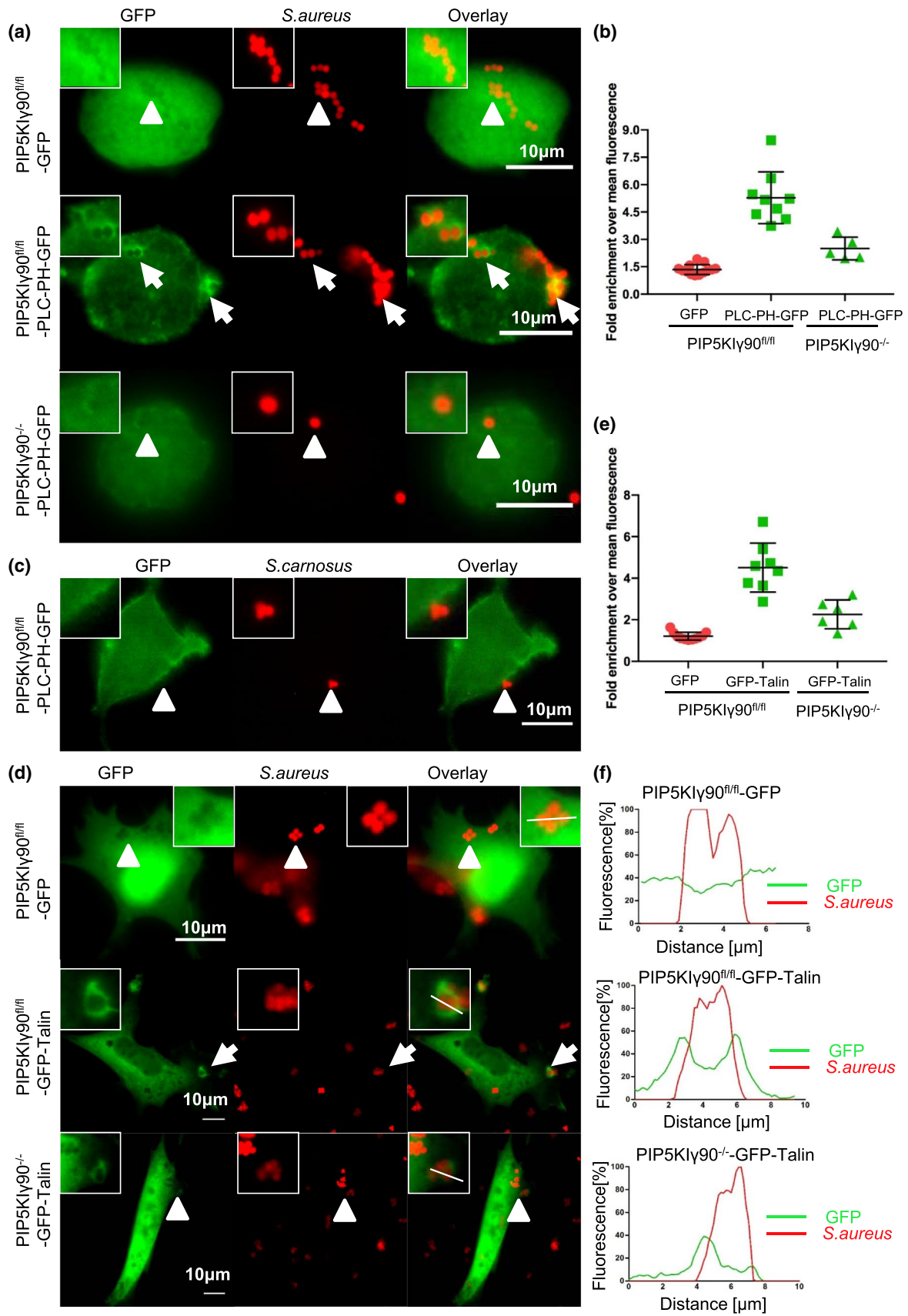


FIGURE 7 Reexpression of active PIP5K1 γ 90 in PIP5K1 γ 90^{-/-} fibroblasts rescues bacterial uptake. (a) PIP5K1 γ 90^{-/-} cells were transfected with plasmids encoding GFP, GFP-PIP5K1 γ 90 wild type, or a kinase-inactive mutant of PIP5K1 γ 90 (PIP5K1 γ 90 D253A), respectively. As a control, PIP5K1 γ 90^{fl/fl} cells were transfected with GFP. Twenty-four hours after transfection, cells were infected with pacific blue-stained and biotin-labeled *Staphylococcus aureus* for 2 hr. After fixation, samples were stained with streptavidin-AlexaFluor647 to mark extracellular bacteria. Arrows indicate examples of intracellular bacteria stained in blue only, whereas arrowheads point to examples of extracellular bacteria stained in blue and red. (b) Experiments were performed as in (a) and the number of total cell-associated (left panel) or intracellular bacteria (right panel) per cell was quantified ($n = 3$ samples; at least 30 cells/sample). Bars show the mean values \pm SEM from three independent experiments. Significance was evaluated by Student's t test. *** $p < .001$, ** $p < .01$, * $p < .05$

FIGURE 8 PIP5K1 γ 90^{-/-} fibroblasts lack local PI-4,5-P₂ production and talin accumulation upon *Staphylococcus aureus* infection. (a) PIP5K1 γ 90^{fl/fl} and PIP5K1 γ 90^{-/-} cells were transfected with GFP or PLC δ -PH-GFP, respectively. Forty-eight hours after transfection, cells were infected for 2 hr with rhodamine-labeled *S. aureus*. Upon fixation, recruitment of GFP or PLC δ -PH-GFP was monitored by confocal fluorescence microscopy. Recruitment of PLC δ -PH-GFP in PIP5K1 γ 90^{fl/fl} cells is indicated by arrows, whereas the absence of GFP or PLC δ -PH-GFP recruitment to bacterial attachment sites is marked by arrowheads. (b) Quantification of GFP fluorescent intensity in samples from (a). The maximum GFP intensity at the infection site was divided by the mean fluorescence intensity of the whole cell. The fold enrichment of 6–8 individual sites is shown. Bars indicate mean fold enrichment and error bars indicate 95% confidence intervals. (c) PIP5K1 γ 90^{fl/fl} cells were transfected with PLC δ -PH-GFP, infected with *S. carnosus*, and evaluated as in (a). The arrowhead indicates the lack of PLC δ -PH-GFP in the few *S. carnosus*-host cell attachment sites. (d) PIP5K1 γ 90^{fl/fl} and PIP5K1 γ 90^{-/-} cells were transfected with GFP or GFP-Talin, respectively. Cells were infected and analyzed by confocal microscopy for GFP or GFP-Talin recruitment as in (a). (e) Quantification of GFP or GFP-talin fluorescence intensity in samples from (d). The maximum intensity at the infection site was divided by the mean fluorescence intensity of the whole cell. The fold enrichment of 6–8 individual sites is shown. Bars indicate mean fold enrichment and error bars indicate 95% confidence intervals. (f) GFP intensity profiles at bacterial attachment sites (indicated by the white line in (d)) highlight the enrichment of GFP-talin in PIP5K1 γ 90^{fl/fl} cells (middle graph) compared with the minor enrichment of GFP-talin in PIP5K1 γ 90^{-/-} cells (lower graph) and the absent enrichment of GFP in PIP5K1 γ 90^{fl/fl} cells (upper graph)



levels during bacterial internalization. Expression of the GFP-tagged PLC δ -PH domain suggests that the PI-4,5-P₂ levels are about 5-fold enriched around cell-bound *S. aureus* in PIP5K1y90^{fl/fl} cells

(Figure 8a,b). Live-cell imaging demonstrated the transient nature of the increase in PI-4,5-P₂, which was most pronounced within 1–5 min upon *S. aureus* contact with the host cell (Figure S5). GFP

alone showed only marginal (1.5-fold) enrichment around *S. aureus* associated with PIP5KI γ 90^{fl/fl} cells (Figure 8a,b). In contrast to the situation in PIP5KI γ 90^{fl/fl} cells, PIP5KI γ 90^{-/-} cells showed strongly reduced (2-fold) accumulation of PLC δ -PH around *S. aureus* (Figure 8a,b). *S. carnosus*, which does not engage integrins, did not influence PLC δ -PH distribution (Figure 8c). As the talin FERM domain is a binding partner of the C-terminal 26 amino acid extension in PIP5KI γ 90, we wondered whether talin accumulation is altered in PIP5KI γ 90^{-/-} cells. To address this question, PIP5KI γ 90^{fl/fl} and PIP5KI γ 90^{-/-} cells were transiently transfected with GFP-tagged talin and, as a negative control, PIP5KI γ 90^{fl/fl} cells with GFP-only, and their expression monitored by Western blotting (Figure S4d). Transfected cells were infected with rhodamine-labeled *S. aureus* for 2 hr. In PIP5KI γ 90^{fl/fl} cells, GFP-talin was recruited upon contact with *S. aureus* (Figure 8d-f). In contrast, GFP-talin accumulation around cell-associated bacteria was almost completely absent in PIP5KI γ 90^{-/-} cells (Figure 8d-f). Combined these results indicate that PIP5KI γ 90 is responsible for local production of PI-4,5-P₂ at the bacterial attachment sites, which is important for talin recruitment to bacteria engaged integrins.

2.9 | PIP5KI γ 90 is critical for maximal FAK activity in response to integrin stimulation

Several signaling processes, including activation of the protein tyrosine kinase FAK as well as phosphorylation of FAK-binding partners, such as paxillin or cortactin, occur upon integrin engagement by *S. aureus* (Agerer et al., 2005). To monitor such downstream signaling events, PIP5KI γ 90^{fl/fl} or PIP5KI γ 90^{-/-} cells were either infected with *S. aureus* for different time periods or left uninfected. Western blot analysis of whole-cell lysates with phospho-tyrosine-specific antibodies revealed that overall tyrosine phosphorylation was reduced in uninfected PIP5KI γ 90^{-/-} compared with the PIP5KI γ 90 expressing cells (Figure 9a). Moreover, PIP5KI γ 90 expressing cells showed a clear increase in tyrosine phosphorylation, and in particular in tyrosine phosphorylation of FAK at the autophosphorylation site Y397, during 2 hr upon *S. aureus* infection (Figure 9a,b). The increase in FAK tyrosine phosphorylation was strongly diminished, and phosphorylation of Y397 was almost completely absent in cells lacking PIP5KI γ 90 (PIP5KI γ 90^{-/-} cells) (Figure 9a,b). These results indicate that maximal activation of tyrosine phosphorylation of FAK downstream of integrin engagement by *S. aureus* requires PIP5KI γ 90 activity. Further reducing FAK activity by pharmacological inhibition with the FAK-specific inhibitor PF431396 led to a complete abrogation of *S. aureus* invasion into wild-type and PIP5KI γ 90^{-/-} MEFs (Figure 9c), suggesting that local PI-4,5-P₂ production by PIP5KI γ 90 is one of several stimulatory inputs for maximal FAK activity. To investigate, if maximal FAK Y397 phosphorylation requires PIP5KI γ 90 activity during physiological stimulation of integrins upon cell-matrix adhesion, PIP5KI γ 90^{fl/fl} or PIP5KI γ 90^{-/-} cells were plated on fibronectin-coated dishes or were kept in suspension. Clearly, cell adhesion to fibronectin stimulated a strong increase in

tyrosine phosphorylation in both PIP5KI γ 90^{fl/fl} and PIP5KI γ 90^{-/-} cells (Figure 9d). Nevertheless, overall tyrosine phosphorylation was slightly elevated in PIP5KI γ 90^{fl/fl} compared with PIP5KI γ 90^{-/-} cells and in particular FAK Y397 phosphorylation, as an indicator of FAK activity, was more pronounced in PIP5KI γ 90^{fl/fl} cells within 90 min of adhesion to fibronectin (Figure 9d). These findings suggest that the local generation of PI-4,5-P₂ by PIP5KI γ 90 is critical for maximal activation of FAK, both in response to fibronectin-binding bacteria as well as during cell adhesion to a fibronectin matrix. Therefore, the reduced activation of FAK in the absence of the PIP5KI γ 90 isoform can help to explain the impairment in the integrin-mediated internalization of *S. aureus*.

3 | DISCUSSION

Although phosphoinositides make up only a small portion of the lipids present within membranes, they are critical for several cellular processes via regulating local recruitment and activation of PIP-binding proteins. Here we demonstrate that PI-4,5-P₂ is enriched at the sites, where host cell integrins are engaged by *S. aureus* via its fibronectin-binding adhesin FnBP. FnBP-mediated contact is sufficient to trigger increased PI-4,5-P₂ levels, which is due to the local recruitment of the 90 kDa isoform of PIP5KI γ . Murine fibroblasts lacking PIP5KI γ 90 are impaired in the integrin-mediated uptake of bacteria suggesting a positive contribution of local PI-4,5-P₂ generation to internalization of *S. aureus*. Indeed, known signaling enzymes downstream of integrin engagement, in particular the focal adhesion kinase, show reduced tyrosine phosphorylation upon bacterial infection of PIP5KI γ 90-deficient cells. As the presence and activity of FAK are required for *S. aureus* internalization (Agerer et al., 2005), diminished FAK activity in PIP5KI γ 90^{-/-} cells can explain the observed phenotype.

It is well established that PI-4,5-P₂ generated by type I PIP5K at the plasma membrane is a critical regulator of constitutive endocytosis (Posor et al., 2015). In particular, the recruitment and functionality of the clathrin adaptor complex AP2 depend on the interaction of AP2 subunits with PIP5K and PI-4,5-P₂ (Padron et al., 2003). Moreover, increased PI-4,5-P₂ levels at the plasma membrane upon overexpression of PIP5K is important for tubule formation during clathrin-independent and dynamin-dependent endocytosis (Soriano-Castell et al., 2017). In the case of vesicular stomatitis virus (VSV), a virus internalized via clathrin-mediated endocytosis, depletion of PI-4,5-P₂ interferes with viral internalization (Vazquez-Calvo et al., 2012). PIP5KI α appears to be the major PIP5KI family member providing PI-4,5-P₂ during clathrin-mediated endocytosis (Antonescu et al., 2011), whereas PIP5KI γ seems to contribute to clathrin-mediated endocytosis in specific cell types only. Indeed, previous studies have shown that specific ablation of the PIP5KI γ 90 isoform in nonneuronal cell types, including murine fibroblasts, does not interfere with clathrin-mediated endocytosis of different plasma membrane receptors (Legate et al., 2011). These findings suggest that PIP5KI α and the remaining PIP5KI isoforms are sufficient to allow regular levels of endocytosis in PIP5KI γ 90^{-/-} cells and point

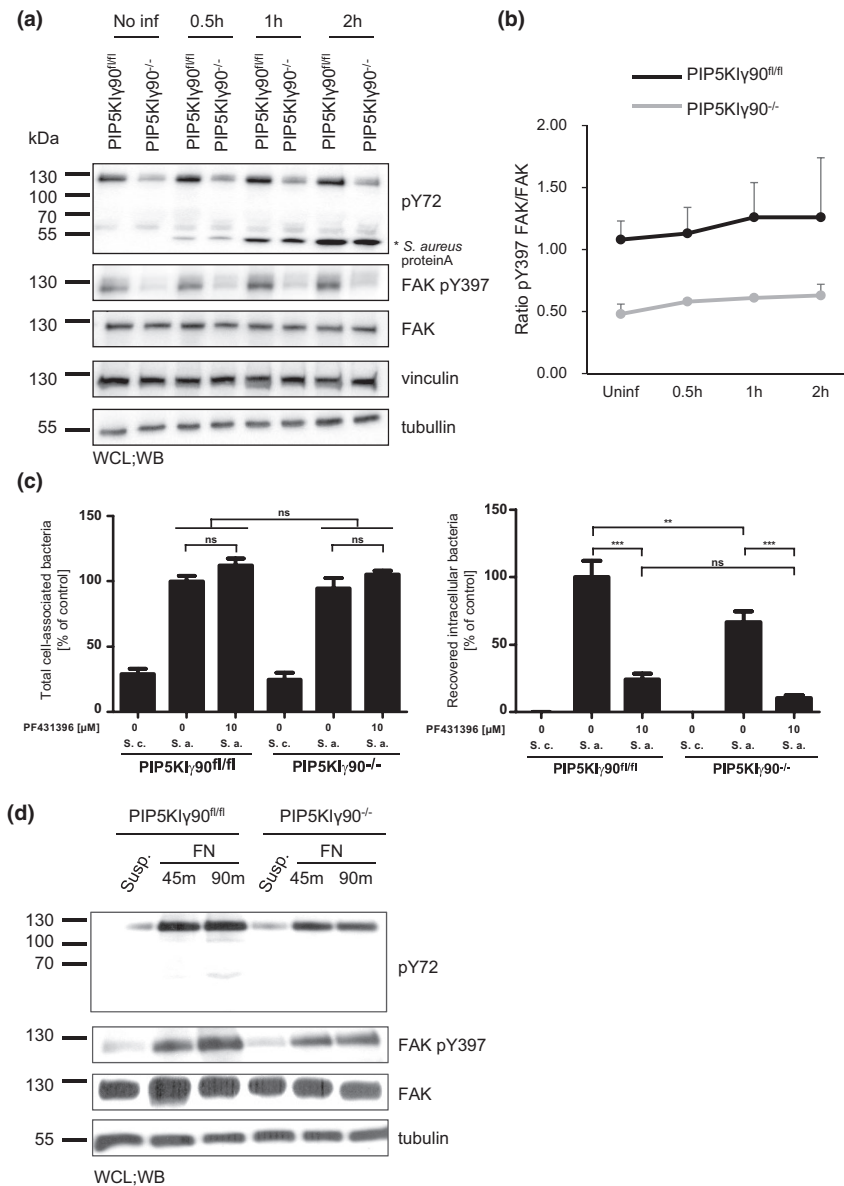


FIGURE 9 PIP5K1 γ 90 is critical for maximal FAK activation in response to integrin stimulation. (a) PIP5K1 γ 90^{fl/fl} and PIP5K1 γ 90^{-/-} cells were serum-starved overnight and then incubated in a suspension medium for 45 min. 1.5×10^6 cells were seeded on poly-L-lysine-coated 6 cm dishes and incubated for 1 hr. Then the cells were infected with fibronectin-coated *Staphylococcus aureus* at MOI 100. At indicated time points, whole-cell lysates (WCL) were prepared. WCLs were analyzed by Western blotting with monoclonal pan-phosphotyrosine antibodies (upper panel), a polyclonal phospho-specific antibody recognizing the phosphoY397 (pY397) form of FAK (second panel), a polyclonal rabbit anti-FAK antibody recognizing total FAK (third panel), a monoclonal antibody against vinculin (fourth panel), or a monoclonal antibody against tubulin (lowest panel). The signal derived from *S. aureus* protein A, which binds the secondary antibody and which increases over time due to bacterial growth, is indicated by (*). (b) Signals obtained from two independent experiments as in (a) were analyzed by densitometry and the intensity ratio of p397FAK versus total FAK at different time points is plotted. (c) Cells were seeded and infected as in (a) with *S. aureus* or *S. carnosus* at an MOI = 20 for 2 hr in a growth medium in the presence of 10- μ M FAK inhibitor PF431396 or DMSO. The total cell-associated and recovered intracellular bacteria were quantified by gentamicin protection assays. The bars show the mean \pm SEM of three independent experiments. Values were normalized to PIP5K1 γ 90^{fl/fl} cells infected with *S. aureus* (control). Significance was evaluated by one-way ANOVA followed by Bonferroni post hoc test. *** $p < .001$. (d) Cells as in (a) were kept in suspension (susp.) or seeded onto fibronectin (15 μ g/ml)-coated 6 cm dishes (1.5×10^6 cells/dish) for the indicated times. WCLs were analyzed by Western blotting with antibodies as indicated in (a)

to other functions of PIP5K1 γ 90, which might relate to the integrin-mediated uptake of *S. aureus*.

Besides regulating endocytosis, PI-4,5-P₂ is an important stimulator of actin cytoskeleton dynamics (Mandal, 2020; Saarikangas

et al., 2010). At focal adhesion sites, several integrin- and actin-associated proteins have PI-4,5-P₂-binding capability and help to connect integrins to the actomyosin cytoskeleton. More precisely, the integrin-associated proteins talin, kindlin, vinculin, FAK, as well

as α -actinin belong to the core focal adhesion proteins, which are responsive to increased PI-4,5-P₂ levels (Janmey, 1994; Janmey et al., 2018; Kelley et al., 2020; Toker, 2002). In this regard, it has been demonstrated that focal adhesion recruitment of talin and vinculin, but not kindlin, is affected in PIP5K1 γ 90-deficient cells (Legate et al., 2011). As vinculin is not involved in the uptake of *S. aureus* (Borisova et al., 2013), a reduced integrin-association of talin, due to the absence of PIP5K1 γ 90, might contribute to a diminished internalization of bacteria. PI-4,5-P₂ binding to the talin head domain is required to release intramolecular inhibitory constraints from this ~280 kDa protein and to allow proper orientation of talin at the inner leaflet of the plasma membrane (Dedden et al., 2019; Elliott et al., 2010; Goksoy et al., 2008; Goult et al., 2013). As a consequence, the recruitment and the spatial orientation of talin at integrin-rich focal adhesion sites are impaired in PIP5K1 γ 90^{-/-} cells, resulting in slower incorporation of talin into new focal adhesion sites (Legate et al., 2011). Indeed, our results demonstrate that PI-4,5-P₂ production by PIP5K1 γ 90 is critical to mobilize talin to newly formed integrin clusters beneath fibronectin-bound bacteria.

In the case of *Bartonella henselae*, a Gram-negative, facultative intracellular bacterial pathogen, integrins, and integrin-associated proteins including talin and FAK are required during host cell invasion (Truttmann et al., 2011). *B. henselae* binds endothelial cells and injects into them a panel of bacterial effector proteins to induce a peculiar F-actin-based structure, the so-called invasome (Dehio et al., 1997). While *B. henselae* does not seem to bind integrins, integrin β 1 is nevertheless essential for invasome formation, which in turn mediates the engulfment of the bacteria. Interestingly, in this context, talin is important for integrin activation via inside-out signaling, which is a prerequisite to allow *B. henselae*-induced formation of invasomes (Truttmann et al., 2011). This is in line with the known function of talin during integrin activation, as the talin head domain associates with an NPXY motif in the cytoplasmic tail of integrin β subunits (Calderwood et al., 1999; Tadokoro et al., 2003). Talin binding separates the intracellular domains of the integrin α and β subunits. Thereby, talin triggers the active, extended conformation of the extracellular domains, which allows ligand binding (Shattil et al., 2010). Though talin might be involved in integrin inside-out signaling during *B. henselae* uptake, integrin activation in response to cell-matrix adhesion does not seem to be compromised in PIP5K1 γ 90-deficient cells (Legate et al., 2011). Furthermore, we do not observe an altered binding of fibronectin-coated *S. aureus* to PIP5K1 γ 90^{-/-} murine fibroblasts suggesting that talin-mediated inside-out activation of integrins is not critical for this process or that such a process does not depend on PI-4,5-P₂ generated by the PIP5K1 γ 90 isoform.

Interestingly, recent findings have highlighted the role of PI-4,5-P₂ during activation of the cytoplasmic protein tyrosine kinase FAK (Feng & Mertz, 2015; Goni et al., 2014; Zhou et al., 2015). Similar to talin, FAK harbors an amino-terminal FERM domain, which interacts via basic amino acid residues with acid membrane phospholipids, in particular PI-4,5-P₂. In the absence of PI-4,5-P₂, the FAK FERM domain binds in *cis* to the FAK kinase domain as part of

an intramolecular autoinhibition mechanism. Once FAK is recruited to ligand-bound integrins, the local presence of PI-4,5-P₂ can lead to a reorientation of the FERM domain, thereby releasing the inhibitory *cis*-interaction with the kinase domain (Goni et al., 2014; Herzog et al., 2017; Zhou et al., 2015). As a result, FAK enzyme activity, including autophosphorylation of a critical tyrosine residue (FAK Y397) located between the FERM and the kinase domain, initiates a series of further tyrosine phosphorylation events (Sulzmaier et al., 2014). Our observation of reduced FAK tyrosine phosphorylation, and in particular diminished phosphorylation of FAK Y397, in PIP5K1 γ 90^{-/-} murine fibroblasts upon *S. aureus* infection or upon cell adhesion to fibronectin indicates that this lipid kinase isoform is critical for maximal FAK activity downstream of integrin $\alpha_5\beta_1$. Interestingly, FAK has been shown to contribute to increased PIP5K1 γ 90 tyrosine phosphorylation, which in turn further reinforces the recruitment of PIP5K1 γ 90 to integrin-initiated focal adhesion sites (Kong et al., 2006; Ling et al., 2003). Together with our findings, these previous results suggest the existence of a positive feedback loop involving local production of PI-4,5-P₂ by PIP5K1 γ 90 in response to integrin engagement, recruitment, and full activation of FAK, which then could stimulate further enrichment of PIP5K1 γ 90 at these sites (Figure S6). Thereby, *S. aureus* hijacks an existing cellular machinery to convert integrins, adhesion receptors specialized in forming mechanically resilient connections to extracellular matrix proteins, into efficient endocytotic devices.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacteria

Escherichia coli Nova Blue was cultured in Lysogeny Broth (LB) medium at 37°C. *S. aureus* Cowan and nonpathogenic *S. carnosus* TM300 were cultured in Tryptic Soybean Broth medium (TSB; BD Biosciences, Heidelberg, Germany) at 37°C. For the infection, *S. aureus* and *S. carnosus* were grown to reach exponential growth phase, washed twice with PBS, and used to infect cells at a multiplicity of infection (MOI) 20 for gentamicin protection assay or MOI 30 for microscopic evaluation of extra- and intracellular bacterial staining. A GST-FnBP fusion protein was produced and purified from *E. coli* as described (Hoffmann et al., 2010).

4.2 | Cell culture

Human embryonic kidney 293T cells (293 cells) were cultured in DMEM supplemented with 10% calf serum (CS) at 37°C in 5% CO₂. HeLa cells were cultured in DMEM with 10% FCS. MEFs from PIP5K1 γ 90 knockout mouse embryos (PIP5K1 γ 90^{-/-} cells) and control PIP5K1 γ mouse embryos (PIP5K1 γ 90^{fl/fl} cells) were isolated and immortalized as described (Legate et al., 2011). MEFs were cultured in DMEM with 10% fetal calf serum (FCS) supplemented with non-essential amino acids and sodium pyruvate on gelatine-coated (0.1%

gelatine in PBS) cell culture dishes. All cell lines were subcultured every 2–3 days and were regularly checked for the absence of mycoplasma.

4.3 | DNA constructs and cell transfection

Expression constructs encoding PLC δ -PH-GFP, Akt-PH-GFP, Btk-PH-GFP, and GFP-OSBP-PH were kindly provided by Tamás Balla (NIH, Bethesda, MD), pRKGFP-Talin was kindly provided by Reinhard Fässler. The pDNR-Dual-myrINPP5J construct was generated by PCR amplification using pCMV-SPORT6-hPIPP (clone IRAKp961K1378Q2 from RZPD, Berlin, Germany) as template and primers 5'-GAAGTTATCAGTCGACATGGGATGTATAAAATCAAAA GGGAAAGACAGCGCGGA GCAGCGCAAAGACAA-3' and anti-5'-TAGAAAGCTTCCGTCCTGAAGGCAAAGT-3'. The resulting PCR fragment was cloned into pDNR-Dual via Sall/HindIII sites and recombinant vector was transferred into pLPS-3'GFP (Clontech) and pLPS-3'Cerulean by Cre-mediated recombination to generate pLPS-3'myrINPP5J-GFP and pLPS-3'myrINPP5J-Cerulean. The enzymatic inactive INPP5J D153A (myrINPP5J-ia) construct was generated by site-directed mutagenesis using the pDNR-Dual-myrINPP5J as a template and primers sense 5'-GGAATACACAGTCAGCGCTCACAAGCC TGTGGC-3' and anti-5'-GCCACAGGCTTGTGAGCGCTGACTGTGT ATTCC-3', then was transferred by Cre-mediated recombination into pLPS-3'GFP and pLPS-3'Cerulean to generate pLPS-3'myrINPP5J-ia-GFP and pLPS-3'myrINPP5J-ia-Cerulean. pEGFP-C1-hPIP5K1 γ 90 has been described previously (Legate et al., 2011). The hPIP5K1 γ 90 D253A mutant was generated by site-directed mutagenesis using primers sense 5'-GTTCCGCCCTCAAGGGCTCCACCTACAAGAGGCG AGCCAGCAAG-3' and anti-5'-GGCTCGCCGCTTGTAGGTGGAGCC CTTGAGGGCGAACTTGTAGGTG-3'. The pcDNA3.1-mKate-CAAX construct was generated by PCR amplification using pLPS-3'mKate as a template and primers 5'-CTCGGATCCGCCATGGTGTCTAAGGG CG-3' and anti-5'-GCCTCGAGTTACATCACCACGCAGGGGAGGCC CATGCAGCCCTGATTAAGT TTGTGCCCCAG-3'. The resulting PCR fragment was cloned into pcDNA3.1 via BamHI/XhoI sites.

For the transfection of 293 cells, standard calcium phosphate coprecipitation with 1–5 μ g of plasmid DNA for each 10 cm culture dish was used. Fibroblasts were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were employed in infection experiments 24–48 hr after transfection.

4.4 | Genotyping PCR

Genomic DNA was isolated from PIP5K1 γ 90^{-/-} and control MEF cells using Genomic DNA Mini Kit (Invitrogen) and then was used as a template to perform PCR. Primers used in this experiment were sense 5'-TACTAACTGCTTCCCGCTGCTGC-3' and anti-5'-GTTTTCTGTGCTTGTGCTGC-3'.

4.5 | Gentamicin protection assay

2×10^5 293 cells or 5×10^4 MEFs were seeded into poly-L-lysine-coated 24-well plates. Cells were infected at MOI 20 for 2 hr at 37°C and 5% CO₂. To evaluate the number of intracellular bacteria, the medium was carefully replaced with a fresh medium containing 50 μ g/ml gentamicin. After incubation for 1 hr at 37°C, intracellular bacteria were released by treatment with 0.5% saponin for 15 min at 37°C. Released bacteria were diluted in PBS and plated on TSB agar plates to determine the colony-forming units (cfu), which resemble the "Recovered intracellular bacteria." In parallel samples, infected cells were gently washed with PBS, lysed with 0.5% saponin without prior gentamicin treatment and dilutions were plated on TSB agar plates. In that way, the "total cell-associated bacteria" were enumerated.

4.6 | Extra- and intracellular bacteria staining and fluorescence microscopy examination

For PIP5K1 γ 90^{-/-} and control MEF cells, 3×10^4 cells for each cell line were seeded on poly-L-lysine-coated, acid-washed glass coverslips in a 24-well plate. The next day, cells were infected with *S. aureus* at MOI 30 for 2 hr, then washed twice with PBS^{+/+} (1 \times PBS containing CaCl₂ and MgCl₂) and fixed with 4% paraformaldehyde (PFA) for 20 min at RT. After this, cells were incubated in a blocking buffer (PBS^{+/+} plus 10% FCS) for 10 min. Extracellular bacteria were detected by rabbit polyclonal α -staphylococcal serum diluted in blocking buffer (45 min at RT). Afterward, samples were washed three times with PBS^{+/+} and incubated with goat α -rabbit IgG-Cy5 (Jackson ImmunoResearch) in the dark for 30 min. Then, cells were washed three times and permeabilized by 0.5% Triton/PBS. Ten minutes later, cells were washed three times with PBS^{+/+}, blocked for 10 min, and incubated with rabbit polyclonal α -staphylococcal serum at RT. Forty-five minutes later, samples were washed three times with PBS^{+/+} and incubated with goat α -rabbit IgG coupled to Cy2 in the dark for 30 min. Finally, after three washes with PBS^{+/+}, the coverslips were embedded in mounting medium (DaKo, Glostrup, Denmark) on glass slides and sealed with nail polish. Images were acquired with a Leica AF6000LX fluorescence microscope and processed with ImageJ.

For the experiment performed in 293 cells, 2×10^5 transfected cells were seeded on poly-L-lysine-coated glass coverslips in a 24-well plate. Two hours later, cells were infected with pacific-blue stained and biotin-labeled *S. aureus* at MOI 30 for 2 hr, then washed twice with PBS^{+/+} and fixed with 4% PFA for 20 min at RT. After this, cells were incubated in a blocking buffer solution for 10 min. Afterward, cells were incubated with streptavidin-AlexaFluor647 for 1 hr at RT in the dark. Finally, after three washes with PBS^{+/+}, the coverslips were transferred to glass slides, embedded in mounting medium, and sealed with nail polish. Images were acquired with a Leica AF6000LX fluorescence microscope.

For immunofluorescence staining of transfected PIP5K1 γ 90^{-/-} cells, 5 × 10⁴ cells were seeded on poly-L-lysine-coated coverslips in a 24-well plate. The next day, cells were transfected with indicated plasmid DNA. Twenty-four hours after transfection, cells were infected with pacific-blue stained and biotin-labeled *S. aureus* at MOI 30 for 2 hr. After the infection, the same staining and measuring procedure as described above for the transfected 293 cells were used.

For cell membrane staining, cells were fixed with 4% PFA, washed three times in PBS and incubated for 10 min in blocking buffer. Cells were stained with CellMask Orange Plasma membrane stain (Invitrogen, cat. No.: C10045) diluted in blocking buffer at a final concentration of 5 μ g/ml for 10 min.

4.7 | Live cell imaging

Cells were seeded into poly-L-lysine-coated 3.5 cm dishes (with integrated coverslip), for example, 5 × 10⁴ cells/dish. The next day, exchange of DMEM medium with colorless DMEM and preparation of the CLSM (heating of incubation chamber to 37°C and connection of CO₂ supply) followed. If the bacterial infection was investigated, cells were directly infected with the fluorescence-stained *S. aureus* at MOI 60. First of all, appropriate cells were searched (e.g., the transfected cells). Then the settings were adjusted and finally, the movie was started to record. The interval in this study was 3 min. Images and movies were analyzed by LAS software and Image J.

4.8 | Quantification of surface integrin expression by flow cytometry

Integrin α_5 (clone 5H10-27(MFR5)) and integrin α_v (clone RMV-7) antibodies were purchased from BD Biosciences. Integrin β_1 (clone HM β 1-1) was obtained from Biolegend and integrin β_3 antibody (clone Hmb3-1) from Millipore (MA, US). Secondary antibodies (biotin-SP-conjugated goat α -rat IgG), streptavidin-FITC, and Rhodamine Red-X-AffiniPure Goat Anti-Armenian Hamster IgG (H+L) were purchased from Jackson ImmunoResearch (West Grove, PA). For quantification of surface integrin expression, suspended fibroblasts were incubated in suspension medium (DMEM containing 0.25% BSA) for 40 min at 37°C. Then, 2 × 10⁵ cells were incubated with appropriate primary antibodies (diluted 1:300) in FACS buffer (5% heat-inactivated FCS, 1% sodium azide in PBS) for 1 hr at 4°C. After washing, secondary antibodies were applied for 1 hr at 4°C, then washed again, samples were analyzed by flow cytometry (LSRII, BD Biosciences).

4.9 | Small interfering RNA transfection

To obtain siRNA-mediated knockdown of PIP5 kinases, HeLa cells instead of 293 cells were used. The siRNA oligonucleotides against PIP5K1 α , PIP5K1 β , and PIP5K1 γ were purchased from Thermo

Scientific. The siRNA transfection was performed according to the manufacturer's instructions. Forty-eight hours after transfection, total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and further used for the quantitative real-time PCR to evaluate the mRNA levels of PIP5K1.

4.10 | qRT-PCR evaluation of PIP5K mRNA levels

Isolation of total RNA from HeLa cells was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany). About 1–2 μ g of total RNA was applied for the reverse transcription. Quantitative real-time PCR was conducted with the sensiMixPlus SYBR Kit (Quantace, Germany) with the following cycle conditions: 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s. Relative expression of PIP5K1 α , β , pan- γ , or γ 90 was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to Livak and Schmittgen (2001). The primers used were: hPIP5K1 α (forward: 5'-GCGTGATGCTCTCATGCAAG-3'; reverse: 5'-GAAGTAGCGGAAGGCAACAG-3'), hPIP5K1 β (forward: 5'-TAGCCAGGAATGGAAGGATG-3'; reverse: 5'-CCAAGGAAGCAGCTTCAAAC-3'), hPIP5K1 γ (forward: 5'-CGTGCAGCGTGGAGATTGTG-3'; reverse: 5'-GCCTGGCTGGCAGTTTCTAC-3'), hGAPDH (forward: 5'-GAAGGTGAAGGTCCGAGTCA-3'; reverse: 5'-TTGAGGTC AATGAAGGGGTC-3').

4.11 | Cell lysis and western blotting

Cell lysis and western blotting were performed as described (Schmitter et al., 2004) with some modifications. Briefly, protein concentration was assessed using Pierce bicinchoninic assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of proteins were loaded on SDS-PAGE gels. Monoclonal antibody against GFP (clone JL-8) was purchased from Clontech and against vinculin (clone hVIN1) was from Sigma-Aldrich. Antibody against β -tubulin (clone E-7, DSHB, University of Iowa) was purified from hybridoma cell supernatants. Polyclonal rabbit antibodies against integrin β 1 (M-106), Cortactin (H-191), and FAK (A-17) were from Santa Cruz Biotechnology. Antibody against FAK pY397 was purchased from Biosource, phosphotyrosine (pY72) monoclonal antibody was purchased from Covance (USA). Goat-antimouse and goat-antirabbit IgG coupled to HRP were purchased from Jackson ImmunoResearch.

4.12 | Fluorescence intensity profile

The corresponding images were digitally processed with Image J (Wayne Rasband, National Institutes of Health, USA) and merged to yield pseudo-colored RGB pictures. The fluorescence intensity at bacterial attachment sites was measured and normalized by the maximal value gained from bacteria staining. The fluorescence percentage was calculated by Graphpad prism 5.

4.13 | Statistics

Infection and flow cytometry experiments were performed at least three times, and data were presented as mean \pm SEM. Differences in adherence and internalization of *S. aureus* were analyzed by unpaired Student's *t* test. In all analyses, a *p* value of $<.05$ was considered to be statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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