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PtpA, a secreted tyrosine phosphatase from *Staphylococcus aureus*, contributes to virulence and interacts with coronin-1A during infection

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Secretion of bacterial signaling proteins and adaptation to the host, especially during infection, are processes that are often linked in pathogenic bacteria. The human pathogen *Staphylococcus aureus* is equipped with a large arsenal of immune-modulating factors, allowing it to either subvert the host immune response or to create permissive niches for its survival. Recently, we showed that one of the low-molecular-weight protein tyrosine phosphatases produced by *S. aureus*, PtpA, is secreted during growth. Here, we report that deletion of *ptpA* in *S. aureus* affects intramacrophage survival and infectivity. We also observed that PtpA is secreted during macrophage infection. Immunoprecipitation assays identified several host proteins as putative intracellular binding partners for PtpA, including coronin-1A, a cytoskeleton-associated protein that is implicated in a variety of cellular processes. Of note, we demonstrated that coronin-1A is phosphorylated on tyrosine residues upon *S. aureus* infection and that its phosphorylation profile is linked to PtpA expression. Our results confirm that PtpA has a critical role during infection as a bacterial effector protein that counteracts host defenses.

The success of *Staphylococcus aureus* as a pathogen and its ability to cause a wide range of disease patterns are the result of its large arsenal of virulence factors that is controlled by a sophisticated network of regulatory molecules (reviewed in Refs. 1 and 2). A number of experiments assessing the invasion and the intracellular survival of *S. aureus* in endothelial and epithelial cells as well as osteoblasts suggest that such events may contribute to the persistence of *S. aureus* during infections such as endocarditis, bovine mastitis, and osteomyelitis (3).

Other work demonstrated that *S. aureus* bacteria possess a high level resistance to neutrophil (4) and macrophage (5) mediated killing, and it has been proposed that professional phagocytes may serve as intracellular reservoirs of *S. aureus* (5, 6). It is nowadays well accepted that the facultative intracellular lifestyle of *S. aureus* contributes to recurrent infections that are frequently observed with this species (7). The pathogen is able to replicate in the phagosome or freely in the cytoplasm of its host cells, and may escape the phagolysosome of professional and nonprofessional phagocytes, subvert autophagy, induce cell death mechanisms, such as apoptosis and pyronecrosis, or may induce anti-apoptotic programs in phagocytes (reviewed in Ref. 8). Earlier work demonstrated that a subpopulation of ingested *S. aureus* bacteria can survive for up to 7 days within macrophages (5, 9), and a number of *S. aureus* global regulators and secreted virulence factors have been identified that contribute to this ability (5, 6, 10–13). However, one strategy utilized by a number of pathogenic bacteria, the secretion of bacterial signaling proteins into target host cells (14, 15), thereby directly modulating host signaling networks, has not yet been studied with *S. aureus*.

Recently, numerous host-pathogen interactions were found to be dependent on pathogen-secreted phosphatases (16–20). Bacterial tyrosine phosphatases catalyze the dephosphorylation of tyrosyl-phosphorylated proteins, which in turn can result in either the propagation or inhibition of phospho-dependent signaling. Whereas bacterial tyrosine phosphatases can be intimately involved in a number of cellular processes, one major theme has become apparent with the involvement of tyrosine phosphatases as secreted effectors with the potential for manipulation of host cell signal transduction pathways (18). Although a detailed picture is yet unavailable, a role of secreted bacterial protein-tyrosine phosphatases during host infection has been identified in different facultative and obligate intracellular pathogens, and the strategies employed by them are currently being elucidated. For instance, the protein-tyrosine phosphatase YopH is a major virulence factor of *Yersinia spp.* that is injected into epithelial cells by the type III secretion machinery of the pathogen. YopH can uncouple multiple signal transduction pathways (21), and in human epithelial cells, YopH dephosphorylates several focal adhesion pro-

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This article contains Figs. S1–S3.

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teins, including p130Cas (Cas), focal adhesion kinase, and paxillin (22–24). Similarly, *Salmonella typhimurium* translocates the low-molecular-weight (LMW)⁵ protein-tyrosine phosphatase (PTP) SptP into epithelial cells to reverse mitogen-activated protein kinase activation (25). Moreover, SptP is required for full virulence in murine models of disease (26). *Mycobacterium tuberculosis* (Mtb) secretes two LMW-PTPs, termed PtpA and PtpB (27). Expression of PtpA in Mtb is up-regulated within monocytes, and PtpA is secreted from Mtb into the host macrophage cytosol and disrupts key components of the endocytic pathway, resulting in the arrest of phagosome maturation (20, 28). Human vacuolar protein sorting 33B (VPS33B), a regulator of membrane fusion, was identified as the cognate substrate of PtpA, and it is assumed that PtpA impairs phagolysosomal fusion in Mtb-infected macrophages by dephosphorylation of VPS33B (20). A Mtb *ptpB* mutant was shown to be impaired in its ability to grow in human macrophages (20), and to display a decreased survival rate in a guinea pig model (29).

A wealth of information has been gained from studies aimed at deciphering the pathophysiological events during *S. aureus*-macrophage infection (reviewed in Ref. 30), but the signaling pathways leading to these adaptations are still poorly understood. The Gram-positive pathogen is known to produce two LMW-PTPs, PtpA and PtpB (31). Earlier work demonstrated that the *S. aureus* PtpA dephosphorylates not only protein tyrosine phosphates, but also protein ribulosamine 5-phosphates as well as free ribuloselysine 5-phosphate and erythroselysine 4-phosphate (32). However, deletion of *ptpA* and/or *ptpB* in *S. aureus* did neither affect the *in vitro* growth kinetics nor the cell wall integrity of the mutants, which led to the assumption that the *S. aureus* Ptp homologs might have some specialized functions during infection (33). Support for this hypothesis is given by our recent observations, indicating that PtpA is secreted during growth of *S. aureus*, albeit of the fact that the protein lacks a clear export pathway signal sequence (34).

Here we demonstrate that PtpA contributes to the intracellular survival capacity of *S. aureus* within macrophages, and participates in the infectivity of this pathogen. Additionally, we show that PtpA is secreted by *S. aureus* upon ingestion by macrophages, and identify potential PtpA interaction partners within this host cell type.

Results

S. aureus PtpA is required for intramacrophage survival

Given the impact of the Mtb PtpA homolog on the persistence capacity of this pathogen within macrophages (35, 36) and the findings that *S. aureus* persists readily within this host immune cell type we wondered whether the PtpA homolog of *S. aureus* might fulfill similar function(s). For this purpose, *ptpA* deletion mutants in *S. aureus* strains Newman, a frequently used laboratory strain, and SA564, a low passage clinical

isolate, were generated. First, we determined the survival rates of *S. aureus* WT and *ptpA* mutants within RAW 264.7 cells at 45 min post-gentamicin treatment (pGt) (Fig. 1A). Already after this short period of time, a significantly smaller proportion of intracellular surviving cells were observed in RAW 264.7 cells infected with the *ptpA* mutants of Newman and SA564, respectively. *Cis*-complementation of the *ptpA* mutants with a functional *ptpA* locus reverted in both cases the intracellular survival rates to levels comparable with WT strains (Fig. 1A). Notably, ingested SA564 bacteria were killed much faster by RAW 264.7 cells than Newman cells. After 45 min pGT, about 80% of the ingested Newman bacteria were still viable and cultivable, whereas this was only the case for 16% of the ingested SA564 bacteria (Fig. 1A). Next, the effect of PtpA on intracellular survival of *S. aureus* Newman in macrophages was determined at a later infection stage (Fig. 1B). Similar to the situation seen at 45 min pGt, significantly reduced survival rates were observed in macrophages infected with the Newman Δ *ptpA* mutant at 22 h pGt when compared with the WT and the *cis*-complemented derivative, respectively, indicating that the survival defect of the *ptpA* mutant in macrophages is maintained over time. *In vitro* growth curves performed with WT and mutant Newman strains excluded that deletion of *ptpA* in *S. aureus* might affect the bacterial growth in suspension (Fig. 1C).

S. aureus PtpA contributes to infectivity of *S. aureus* in a murine abscess model

Because PtpA enhances the survival capacity of *S. aureus* within macrophages (Fig. 1, A and B), we hypothesized that PtpA may affect the infectivity of *S. aureus* *in vivo*. To address this hypothesis, we next assessed the ability of the strain triplet SA564/SA564 Δ *ptpA*/SA564 Δ *ptpA::ptpA* to cause disease in a murine abscess model (37). Consistent with our intramacrophage survival findings (Fig. 1), a significant decrease (about 2-log) in the bacterial loads in liver was detected in mice infected with the Δ *ptpA* mutant as compared with mice challenged with the WT strain (Fig. 2). Mice infected with the *cis*-complemented *ptpA*⁺ derivative SA564 Δ *ptpA::ptpA* displayed significantly higher bacterial loads in liver than in Δ *ptpA* mutant-challenged mice, although the levels remained lower as seen in WT-challenged animals, however, this effect was not statistically significant ($p = 0.215$). These data suggest that PtpA positively contributes to the infectivity of *S. aureus* in mice.

PtpA is secreted intracellularly upon *S. aureus* macrophage infection

We recently demonstrated that PtpA was secreted into the extracellular milieu by *S. aureus* under *in vitro* growth conditions (34). To test whether PtpA might be also secreted by *S. aureus* into the host macrophage, we used the *cis*-complemented *S. aureus* derivative Newman Δ *ptpA::ptpA*_FLAG (34), which facilitates detection of the expressed PtpA by anti-FLAG immunoblotting. Macrophages were infected with the Newman Δ *ptpA* mutant or the *cis*-complemented Newman Δ *ptpA::ptpA*_Flag derivative expressing PtpA__{FLAG}, lysed at different time points pGt, separated from the intracellular bac-

⁵ The abbreviations used are: LMW, low-molecular-weight; PTP, protein-tyrosine phosphatase; VPS, vacuolar protein sorting; pGt, post-gentamicin treatment; GFP, green fluorescent protein; GST, glutathione S-transferase; CorA, coronin-A; Ni-NTA, nickel-nitrilotriacetic acid; TSB, tryptic soy broth; cfu, colony forming unit; m.o.i., multiplicity of infection.

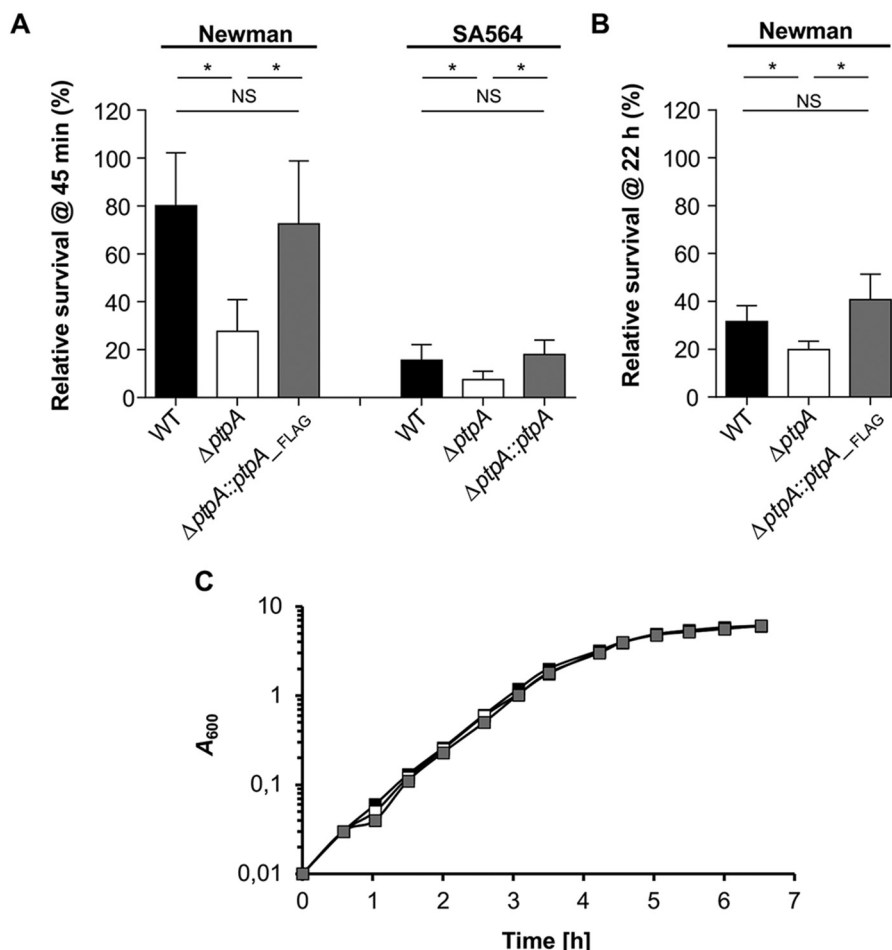


Figure 1. PtpA promotes *S. aureus* survival in macrophages. *A*, *S. aureus* short-term survival in infected macrophages. Cells of *S. aureus* strains SA564 and Newman and their isogenic $\Delta ptpA$ mutants and *cis*-complemented derivatives, respectively, were used to infect RAW 264.7 macrophages (5×10^5 cells/ml) at a m.o.i. of 20 for 1 h at 37 °C followed by 30 min incubation with gentamicin. Macrophages were then incubated for 45 min in complete media supplemented with lysostaphin to kill extracellular bacteria that might be released from lysed macrophages during the successive incubation time, and subsequently lysed in 0.1% Triton X-100. Survival rates are given in relationship to the intracellular bacterial cell numbers seen pGt treatment. Results represent the mean \pm S.D. ($n = 5$). *, $p < 0.05$; NS, not significant (Mann-Whitney-*U* test). *B*, *S. aureus* long-term survival in infected macrophages. RAW 264.7 macrophages (5×10^5 cells/ml) were infected with a m.o.i. of 20 of *S. aureus* strains Newman (black bar), Newman $\Delta ptpA$ (white bar), and Newman $\Delta ptpA::ptpA_Flag$ (gray bar), respectively, and co-incubated for 2 h at 37 °C, followed by a gentamicin/lysostaphin treatment to eliminate external bacteria. Macrophages were lysed with Triton X-100 (0.1%) at 22 h pGt, and bacteria enumerated by plate counting. Survival rates are given in relationship to the intracellular bacterial cell numbers seen immediately after gentamicin treatment. Data represent the mean \pm S.D. ($n = 5$). *, $p < 0.05$; NS, not significant (Mann-Whitney-*U* test). *C*, *in vitro* growth kinetics of the *S. aureus* Newman/ $\Delta ptpA$ / $\Delta ptpA::ptpA_Flag$ strain triplet. Growth of *S. aureus* strains Newman (black symbols), Newman $\Delta ptpA$ (white symbols), and Newman $\Delta ptpA::ptpA_Flag$ (gray symbols) was measured in TSB at 37 °C and 150 rpm. Data represent the mean A_{600} readings at the time points indicated ($n = 3$).

teria and processed for anti-FLAG immunoprecipitation and Western blot analysis. PtpA_{FLAG} could be detected in increasing amounts in macrophage lysates over time upon cell infection with the *ptpA* complemented Newman strain $\Delta ptpA::ptpA_FLAG$, whereas no such signal was observed in Newman $\Delta ptpA$ -infected macrophages (Fig. 3A).

To exclude that the PtpA_{FLAG} signal might originate from lysed bacteria, we transformed the Newman $\Delta ptpA$ mutant and the *cis*-complemented Newman $\Delta ptpA::ptpA_FLAG$ derivative with the green fluorescent protein (GFP) expressing vector pMK4_GFP. Following macrophage infection with these pMK4_GFP harboring derivatives, no GFP signal could be detected in the cleared macrophage lysates after concentration by GFP immunoprecipitation (GFP-trap, Chrommoteck), whereas strong GFP signals were detected in the bacterial pellets that were obtained from the macrophage lysates by centrifugation (Fig. 3B). In the same samples, PtpA_{FLAG} was detected

in the bacterial pellet and after FLAG-immunoprecipitation from the cleared lysates of the Newman $\Delta ptpA::ptpA_FLAG$ + pMK4_GFP-infected macrophages, whereas this signal was not seen in the cleared lysates of macrophages that were challenged with the pMK4_GFP-transformed $\Delta ptpA$ mutant (Fig. 3C). Taken together, these findings indicate that the PtpA_{FLAG} signal detected in the lysate fractions of macrophages infected with the Newman $\Delta ptpA::ptpA_Flag$ derivative was not substantially caused by intracellular bacterial lysis, suggesting a secretion of PtpA into macrophages.

PtpA interacts with several host cell proteins in pull-down experiments

Because PtpA is most likely secreted into host cells, we next assessed whether this bacterial derived phosphatase might interfere with host cell signal transduction pathways. To identify host cell proteins that might interact with PtpA, we

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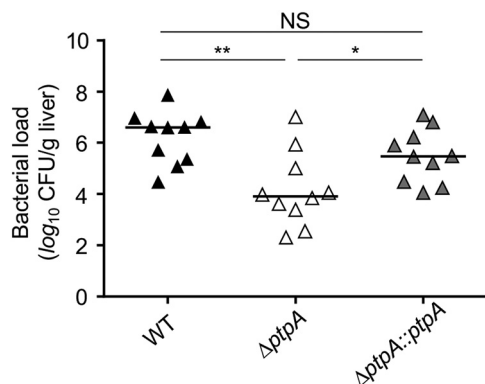


Figure 2. Effect of *ptpA* deletion on infectivity of *S. aureus* SA564 in a murine abscess model. C57BL/6N mice were infected via retroorbital injection with 1×10^7 cells of *S. aureus* strain SA564 (black symbols), SA564 $\Delta ptpA$ (white symbols), and the *cis*-complemented derivative SA564 $\Delta ptpA::ptpA$ (gray symbols), respectively ($n = 10$ per group). Mice were euthanized 4 days post-infection, the livers were removed and homogenized in PBS to determine the bacterial loads. Each symbol represents an individual mouse. Horizontal bars indicate the median of all observations. *, $p < 0.05$; **, $p < 0.01$; NS, not significant (Mann-Whitney-*U* test).

developed a strategy combining the use of the slime mold *Dictyostelium discoideum*, and our PtpA functional mutants. *D. discoideum* is a eukaryotic professional phagocyte amenable to genetic and biochemical studies, and in our case allowing the ectopic expression of PtpA in large culture volumes. In a second approach, we used a “substrate trapping” strategy, based on a methodology used to identify substrates for the *Yersinia* phosphatase YopH in HeLa cells (38), or Mtb PtpA host interactants (20). The latter mechanism-based approach utilized a catalytically defective mutant of PtpA to trap substrate complexes. PTPs contain a cysteine nucleophile (Cys-8 within the highly conserved sequence C-X₅-R) that forms a phosphocysteinyll intermediate during catalysis (39). We hypothesized that a cysteine to serine mutation at position 8 of the *S. aureus* Newman PtpA ORF (PtpA_C8S) would result in a catalytically defective PtpA variant that, like other similar PTP family mutants, might “trap” host substrate proteins by stabilizing the covalent enzyme-substrate complexes. To confirm that the C8S mutation in *S. aureus* PtpA affects its activity, we first tested the recombinant His-tagged versions of PtpA and PtpA-C8S with the substrate *p*-nitrophenylphosphate. Our results demonstrate that phosphatase activity of the *S. aureus* C8S mutant was indeed abrogated (Fig. S1).

Lysates from *D. discoideum* overexpressing PtpA_C8S_{-FLAG} or expressing the FLAG alone as a control, were next incubated with beads coupled to an anti-FLAG antibody. Afterward, beads were extensively washed, and bound proteins were subsequently stripped off and separated by SDS-PAGE before MS analysis. The proteins identified under each condition were compared and purged of those interacting with FLAG alone (Table 1).

The putative interactants of PtpA identified by this approach are involved in different cellular pathways involving cell adherence or endosomal trafficking. Two of them, lysosomal- α -mannosidase ManA and Arf-GTPase-activating protein DDB0167328, likely play a role in cell adherence (40, 41). Lysosomal- α -mannosidases are members of the glycoside hydrolase family 38. They are involved in the catabolism of Asn-linked glycans of glycoproteins and play a vital role in

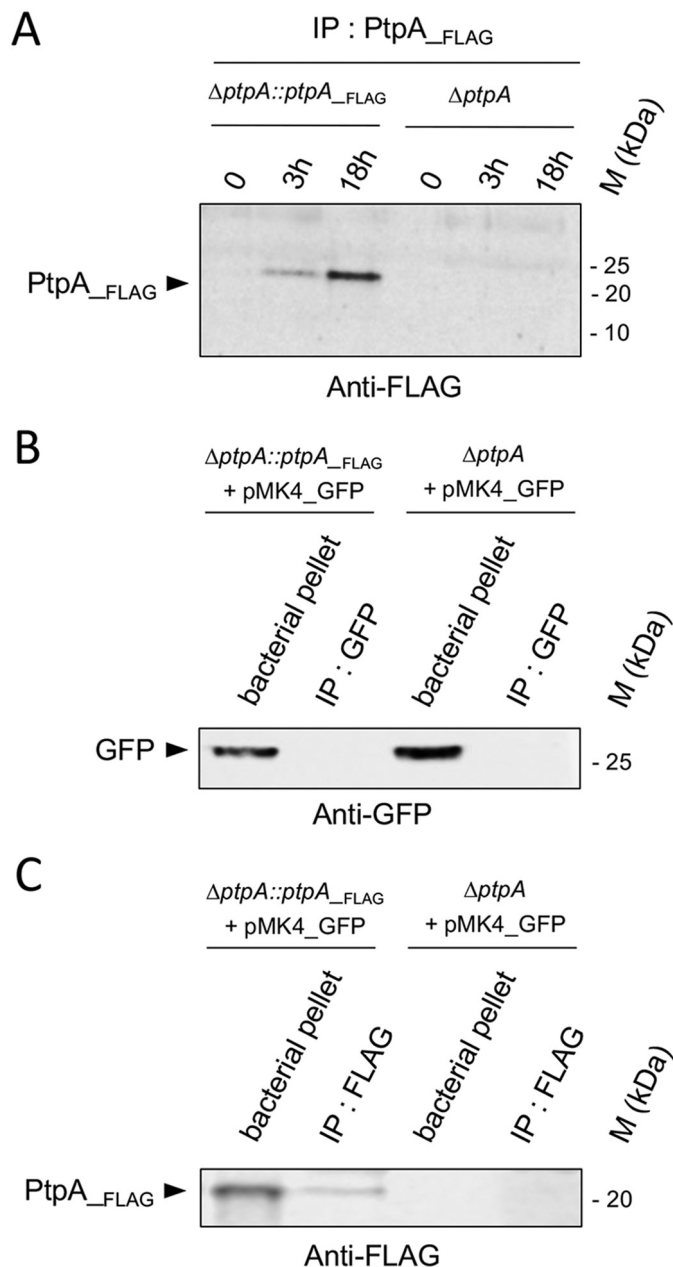


Figure 3. PtpA is secreted in macrophages during infection. A, PtpA secretion in macrophage lysates. RAW 264.7 macrophages (5×10^5 cells/ml) were incubated for 2 h with either *S. aureus* Newman $\Delta ptpA$ cells or *S. aureus* $\Delta ptpA$ cells complemented with a FLAG-tagged *ptpA* (Newman $\Delta ptpA::ptpA_Flag$) at a m.o.i. of 20, and nonphagocytosed bacteria were subsequently removed by gentamicin/lysostaphin treatment. Macrophages were lysed at the time points indicated, centrifuged to eliminate intracellular bacteria, and macrophage lysates were subjected to immunoprecipitation and Western blotting analyses using anti-FLAG antibodies. B and C, control immunoprecipitations to rule out bacterial proteins leaking in macrophage lysates. RAW 264.7 macrophages (5×10^5 cells/ml) were infected with Newman $\Delta ptpA::ptpA_FLAG$ harboring plasmid pMK4_GFP and Newman $\Delta ptpA$ harboring plasmid pMK4_GFP at a m.o.i. of 20, respectively. At 3 h pGt, infected macrophages were lysed in 0.1% Triton X-100, and centrifuged at $14,000 \times g$. The obtained supernatants corresponding to macrophage lysates were immunoprecipitated with GFP-trap beads (Chromotek) (B) or with anti-FLAG antibodies coated on agarose-beads (C), whereas the pellets containing intracellular bacteria were resuspended in an equal amount of PBS with protease inhibitor mixture and lysed in a bead-beater (Retsch, MM400). Immunoprecipitated proteins and bacterial pellets were resolved on SDS-PAGE and detected with an anti-GFP (B) or anti-FLAG (C) antibody. M kDa, molecular markers.

Table 1
Major PtpA interactants identified by mass spectrometry

Protein name	Accession number	Sum PEP score ^a	Coverage ^b	Number of peptides ^{c,d}	Mass
V-type proton ATPase subunit A	P54647	3216	65	30	68.16
V-type proton ATPase subunit B	Q76NU1	454	35	12	54.84
Coronin-A	P27133	435	33	10	49.18
Cathepsin D	O76856	421	19	6	41.09
Vacuolin-A	O15706	196	13	6	66.25
Lysosomal α -mannosidase	P34098	131	7	4	113.36
Arf GTPase-activating protein	Q9Y2X7	110	10	3	64.89
Phox domain-containing protein vps5	Q86IF6	26	9	2	61.92

^a Sum PEP score: sum of $-\log(\text{PEP})$ (PEP: posterior error probability), which is a probability that a Peptide Spectra Matches (PSM) is incorrect. The lower the PEP, the higher the sum PEP score.

^b % of protein sequence coverage.

^c A peptide is identified by one or more PSM corresponding to relevant MS-MS mass spectra leading to the identification of a peptide. A protein is identified by several peptides (# Peptides).

^d The cut-off is validated by the SEQUEST HT algorithm and corresponds to at least two peptides to identify the protein.

maintaining cellular homeostasis, cell adhesion during development, viral infection, or immune response (40). Among several functions, Arf-GTPase activating proteins are notably regulators of specialized membrane surfaces implicated in cell migration involving adhesive structures in which the cell membrane is integrated with the actin cytoskeleton (41). Additionally, proteins related to endosome function and trafficking have been co-immunoprecipitated with PtpA: Vacuolin A, Cathepsin D, and Phox domain-containing protein Vps5. Vacuolin A is a flotillin/reggie-related protein from *Dictyostelium* that oligomerizes for endosome association (42), and Cathepsin D is an aspartic endoprotease that is ubiquitously distributed in lysosomes to degrade proteins and activate precursors of bioactive proteins in pre-lysosomal compartments (43). Vps5 belongs to the family of sorting nexins containing a Phox homology domain and might be a component of the retromer complex. These proteins are involved in regulating membrane traffic and protein sorting in the endosomal system (44). Interestingly, V-ATPase, previously identified as an interactant of *M. tuberculosis* PtpA (28), was captured also in our substrate-trapping assay, suggesting that in *S. aureus* a similar interaction might occur. Moreover, the host protein coronin-A (CorA) was identified as putative PtpA interactant (Table 1). Interestingly, the mammalian homologue coronin-1A (Coro-1A) was reported as being retained on phagosomes containing living Mtb, while being rapidly released from phagosomes containing inactive mycobacteria (45). Furthermore, genetic depletion or RNAi-mediated gene silencing of Coro-1A were later reported to inhibit the survival of mycobacteria within macrophages (46–48).

PtpA interacts with coronin in vitro

As Coro-1A was shown to be important for the survival of mycobacteria in infected macrophages (45–49), we decided to investigate the putative interaction of *S. aureus* PtpA and Coro-1A in more detail. First, we performed GST pull-down assays to verify the interaction between PtpA and *D. discoideum* CorA (Dd_Coro-A) (Fig. 4A). Fusion proteins combining GST and PtpA (PtpA_{GST}) were immobilized onto GSH-agarose beads and incubated with *D. discoideum* cell lysates expressing a myc-tagged-Dd_Coro-A (myc-Dd_Coro-A). Bound proteins were stripped off the beads and subjected to Western blotting analyses with an anti-myc antibody. As displayed in Fig. 4A, myc-Dd_Coro-A was pulled down with the PtpA_{GST}

fusion, but not with GST alone. This observation strongly suggests that PtpA interacts with CorA from *D. discoideum*, thus supporting our MS findings.

Next, we assayed the interaction of the *S. aureus* PtpA with human Coro-1A (Hs_Coro-1A) by using a pull-down assay (Fig. 4B). His-tagged versions of PtpA (PtpA_{His}) and the catalytically inactive PtpA_C8S (PtpA_{C8S_His}) were overexpressed and purified as previously described (34), bound on Ni-NTA-agarose beads and incubated with BL21 lysates expressing the recombinant Hs_Coro-1A protein harboring a GST tag (Hs_Coro-1A_{GST}). As control, PtpA_{His} beads were incubated with BL21 lysates expressing GST alone. Ni-NTA-agarose beads without PtpA were additionally incubated with GST or Hs_Coro-1A_{GST} to confirm that beads alone could not interact with GST fusion proteins (Fig. 4B, upper panel). Protein complexes were pulled down, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane before detection by anti-GST and anti-coronin-1A antibodies, respectively. In this assay, Hs_Coro-1A_{GST} was retained on beads when PtpA_{C8S_His} was present, whereas no signal was seen with the PtpA_{His} version (Fig. 4B, lower panel). Additionally, GST alone did neither in absence nor presence of the His-tagged PtpA fusion proteins bind to the beads (Fig. 4B). Taken together, these data suggest that specific complexes were formed between *S. aureus* PtpA and Coro-1A either with the *D. discoideum* CorA or the human homologue Hs_Coro-1A.

Coronin-1A is phosphorylated on tyrosine residues upon infection

To test whether Coro-1A might serve as a tyrosine-phosphorylated substrate for PtpA *in vivo*, we infected RAW 264.7 cells with *S. aureus* Newman and its isogenic ΔptpA mutant, respectively, and lysed the macrophages for 30 min and 3 h pGt. The murine macrophage Coro-1A homolog (Mm_Coro-1A) was subsequently immunoprecipitated with an anti-Coro-1A antibody, and the tyrosine phosphorylation status of Mm_Coro-1A was determined by Western blotting analyses using anti-phosphotyrosine antibodies. We observed that phosphorylation of Mm_Coro-1A on tyrosine residues was increased upon infection, and this effect seemed to be influenced by PtpA (Fig. 5A). Infection of RAW 264.7 cells with *S. aureus* clearly enhanced the tyrosine phosphorylation signal of Mm_Coro-1A at both time points analyzed. Interestingly, in lysates of Newman

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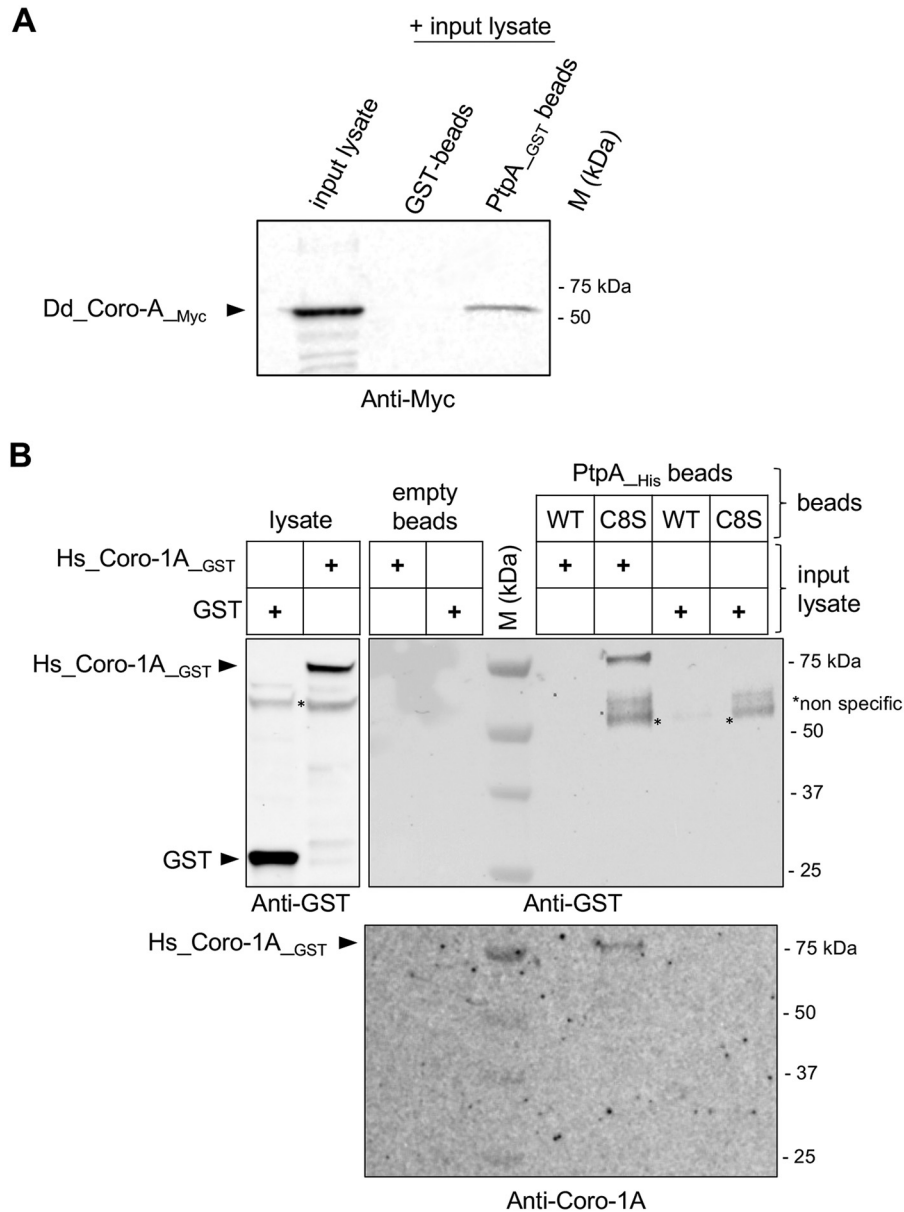


Figure 4. PtpA interacts with Coro-1A *in vitro*. *A*, the interaction between PtpA and *D. discoideum* CorA was confirmed by GST pull-down assay. The indicated GST fusion proteins were expressed in *E. coli*, bound to GSH beads, and then incubated with *D. discoideum* cell lysates expressing myc-tagged-Dd_Coro-1 (Dd_Coro-A_{-myc}). Beads were washed, eluted by boiling, and bound proteins were revealed by Western blot analysis with an anti-myc antibody. *B*, the interaction between PtpA and human Coro-1A was tested by pull-down analysis. His-tagged versions of PtpA (WT) and the catalytically inactive PtpA_C8S (C8S) were constructed, produced, and purified as described under "Experimental procedures." BL21 lysates expressing Hs_Coro-1A_{-GST} or GST alone were prepared and incubated with the PtpA_{-His} derivatives immobilized on Ni-NTA-agarose beads. The bound proteins were eluted and resolved by SDS-PAGE followed by immunoblotting using anti-GST and anti-Coro-1A antibodies. Empty Ni-NTA-agarose beads were used as a control. *, nonspecific signals co-precipitated with PtpA_C8S_{-His}. M kDa, molecular markers.

Δ *ptpA*-infected macrophages, about 2-fold higher phosphotyrosine signals (2.2 ± 0.7 ; $n = 6$) were observed at 3 h pGT than in lysates of RAW 264.7 cells that were challenged with the parental strain Newman. Therefore, our results reveal for the first time a tyrosine phosphorylation of Coro-1A, as previous records of phosphorylation for this protein were related only to Ser-Thr residues (50–52), as well as its PtpA-mediated dephosphorylation.

Coronin-1A is not dephosphorylated *in vitro* by recombinant PtpA

The observation that Coro-1A can be Tyr-phosphorylated, and that its Tyr-phosphorylation status seemed to be influ-

enced by PtpA, prompted us to assess whether Coro-1A might be directly dephosphorylated by PtpA, as previously reported for some Mtb PtpA substrates (20). Thus we tested the ability of PtpA and of the catalytically inactive PtpA derivative PtpA_{D120A} (34) to dephosphorylate the murine variant of Coro-1A. PtpA_{D120A} was chosen for this dephosphorylation assay as it corresponds to the commonly used phosphatase-defective PtpA mutant PtpA_{D126A} in Mtb (20, 28, 53, 54). First, Mm_Coro-1A was purified by immunoprecipitation from *S. aureus* Newman-infected macrophages. Equal amounts of the immunoprecipitated Mm_Coro-1A were then incubated with recombinant PtpA derivatives for 30 min and 1 h, respec-

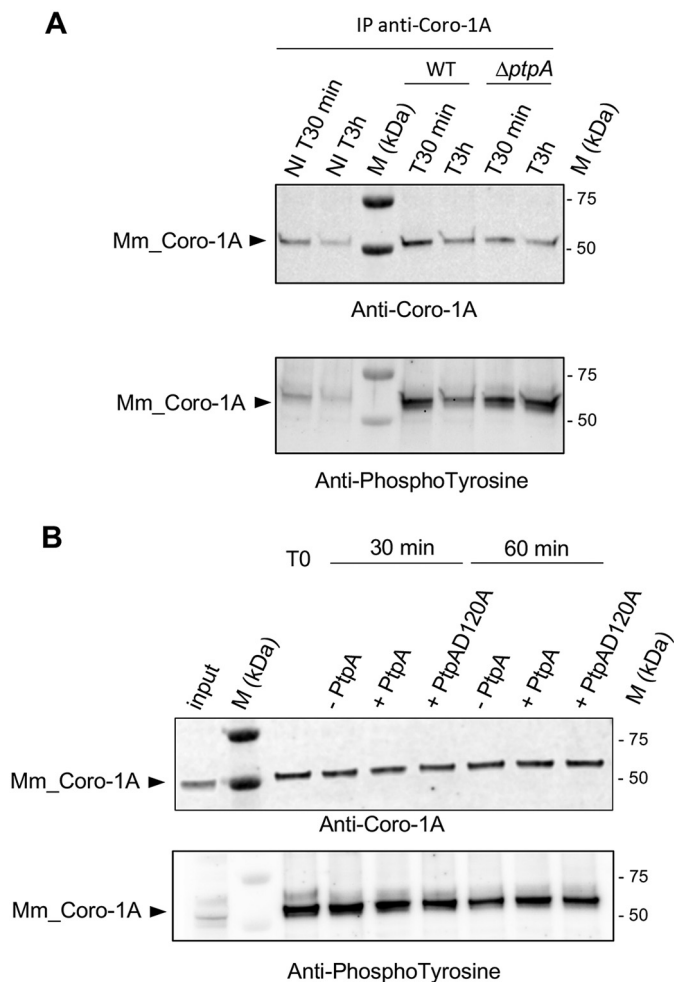


Figure 5. Coro-1A is phosphorylated on tyrosines *in vivo* but is not dephosphorylated by PtpA *in vitro*. *A*, immunoblot analysis with anti-Coro-1A (upper panel) or anti-phosphotyrosine antibodies (bottom panel) of immunoprecipitated (IP) endogenous Coro-1A (Mm_Coro-1A) from lysates of RAW 264.7 macrophages infected with cells of *S. aureus* Newman (WT) or the isogenic ΔptpA mutant. Noninfected macrophages and infected macrophages were treated with gentamicin for 30 min and subsequently incubated with lysostaphin to kill extracellular bacteria that might be released from lysed macrophages during the successive incubation time. Noninfected and infected macrophages were lysed 30 min (NI T30 min and T30 min) and 3 h (NI T3 h and T3 h) pGt treatment. *B*, immunoprecipitated Mm_Coro-1A from lysates of macrophages infected with strain Newman for 30 min pGt were incubated in the absence (–) or presence (+) of 2 μg of PtpA_{HIS} or PtpA-D120A_{HIS} at 37 °C for the time points indicated (T0, 30 and 60 min). Proteins were resolved by SDS-PAGE and probed with anti-Coro-1A (upper panel) or anti-phosphotyrosine (bottom panel) antibodies on the same blot. Contents of Mm_Coro-1A and Tyr-phosphorylated Mm_Coro-1A in lysates of *S. aureus*-infected macrophages prior to concentration by IP are indicated in the input lane (input). *M kDa*, molecular markers.

tively. Proteins were subsequently separated by SDS-PAGE and electrotransferred to a membrane. The Coro-1A and Tyr-phosphorylation signals were revealed using anti-Coro-1A and anti-phosphotyrosine antibodies, respectively (Fig. 5). However, no clear reductions in the phosphorylation levels of Mm_Coro-1A were observed after incubation with either PtpA or the catalytically inactive PtpA_{D120A}. All together, these data indicate that Mm_Coro-1A is a bona fide substrate for tyrosine phosphorylation, and that PtpA seems to affect Coro-1A Tyr-phosphorylation in an indirect manner. Notably, a similar observation was made with the Mtb PtpA, which is able to interact with

the V-ATPase subunit H without using this protein as a catalytic substrate (28).

To identify putative Tyr-phosphorylation sites of Coro-1A, we next performed an *in silico* analysis with the ORFs of the Coro-1A variants used in this study. The alignment of the amino acid sequences of the three Coro-1A homologues identified 94% of homology between the Mm_Coro-1A and Hs_Coro-1A ORFs, whereas the Dd_Coro-1A homologue shared only 38% of homology with the human Coro-1A (Fig. S2). Despite the comparably low degree of conservation between Dd_Coro-1A and the two mammalian Coro-1A variants, four Tyr-residues were identified as conserved among the three species. A subsequent alignment of 25 Coro-1A homologs of various eukaryotes including protists, fungi, and animalia confirmed a very high conservation of these four Tyr residues (Fig. S3), supporting the idea that these sites might be involved in the Tyr-mediated phosphorylation of this protein.

In a first attempt to identify the host kinase responsible for Coro-1A Tyr-phosphorylation upon *S. aureus* infection, we tested the spleen tyrosine kinase Syk on Mm_Coro-1A Tyr-phosphorylation, as Syk is highly expressed in RAW 264.7 cells (55), and implicated to play a pivotal role in macrophage-mediated inflammatory responses (56). Moreover, one of the human coronin homologs, coronin-1C (sharing 62% of identity to Hs_Coro-1A at the amino acid level), was previously identified as ligand of Syk in B-cells (57). However, we failed to detect any tyrosine phosphorylation signal on Coro-1A after co-incubation with Syk in our *in vitro* phosphorylation assays (data not shown), indicating that Syk is not the kinase responsible for Tyr-phosphorylation of Coro-1A.

Discussion

Our results provide the first interactor candidate identification of host partners of the secreted phosphatase PtpA, and its involvement in the process of infection and intracellular survival of *S. aureus*. The macrophage survival and infection data suggest an important role for PtpA during infection. Our co-immunoprecipitation studies indicate PtpA interacts with a number of host factors including Coro-1A, and revealed that this actin-binding protein can be phosphorylated at tyrosine residues. Together, these findings suggest a role for PtpA as modulator of the host immune response, particularly after uptake of *S. aureus* by macrophages. As the impact of Tyr-phosphorylation on Coro-1A function/activity was not studied yet, we can only hypothesize that phosphorylation of the Coro-1A Tyr residue(s) might affect the intracellular distribution of this actin-binding protein within macrophages, as it has been suggested for serine/threonine-mediated phosphorylation of coronin-1. Indeed, protein kinase C-dependent coronin-1 phosphorylation on Ser and Thr residues was identified as an important mechanism to modulate the intracellular distribution of this protein during phagolysosome maturation. Earlier work studying the phagocytosis of opsonized zymosan particles by HL-60 leukemia cells demonstrated that phosphorylation of coronin-1 by protein kinase C triggered the dissociation of the actin-binding protein from nascent phagosomes (51). Another study performed with mycobacteria-infected macrophages showed that coronin-1 accumulated on bacteria-loaded phagosomes, and

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Table 2

Strains, plasmids, and primers used in this study

Strain or plasmid	Relevant genotype or characteristic(s) ^a	Ref. or source
<i>S. aureus</i>		
Newman	Laboratory strain (ATCC 25904), wildtype	
Newman $\Delta ptpA$	Newman $\Delta ptpA::lox66-aphAIII-lox71$; Kan ^r	34
Newman $\Delta ptpA::ptpA_Flag$	Newman $\Delta ptpA$ derivative <i>cis</i> -complemented with pEC1_ptpA_Flag; Em ^r	34
SA564	Clinical isolate, wildtype	60
SA564 $\Delta ptpA$	SA564 $\Delta ptpA::lox66-aphAIII-lox71$; Kan ^r	This study
SA564 $\Delta ptpA::ptpA$	SA564 $\Delta ptpA$ derivative <i>cis</i> -complemented with pEC1_ptpAc; Em ^r	This study
<i>E. coli</i>		
IM08B	SA08B Ω P _{N25} - <i>hdsS</i> (CC8-1) (SAUSA300_0406) of NRS384 integrated between the <i>essQ</i> and <i>cspB</i> genes	62
<i>D. discoideum</i>		
DH1-10	Axenic, uracil auxotroph <i>D. discoideum</i> strain	64
Plasmids		
pDXA	<i>D. discoideum</i> vector	63
pFL1290	pDXA-3 × myc-Coronin-A corresponding to pDXA vector with a 1338-kb fragment covering the ORF for the <i>D. discoideum</i> coronin-A (<i>corA</i> ; Gene ID: DDB_G0267382; NCBI reference sequence: XM_642251.1) fused to 3 × myc tag.	This study
pDXA_PtpA-C8S-Flag	pDXA_PtpA-C8S-FLAG corresponding to pDXA vector with a 0.8-kb fragment covering the ORF for the <i>S. aureus</i> <i>ptpA</i> gene fused to 3 × FLAG tag.	This study
pEC1	pUC19 derivative containing the 1.45-kb ClaI <i>erm</i> (B) fragment of Tn551	61
pEC1_ptpAc	pEC1 with a 0.8-kb fragment covering the C-terminal part of ORF RT87_RS09705 and the putative terminator region (NCBI reference sequence: NZ_CP010890.1).	This study
pETPhos_PtpA_C8S	Modified from pETPhos_PtpA (34)	This study
pCDFDuet GST Cter up1	Modified from Novagen pCDFDuet-1	This study
pCDFDuet-Coro-1A-GST	pCDFDuet-GST Cter Up1 with a 1.4-kb fragment covering the ORF for the human Coro-1A (NCBI reference sequence: NC_000016.10) optimized for bacterial expression and fused to GST.	This study
Primers		
OL989-5'-BamHIx2 (forward)	GGATCCGGATCCATGCTCTAAAGTAGTCCGCTAGTAGTAAA	This study
OL990-3'-XhoIx2-stop (reverse)	CTCGAGCTCGAGTTAGTTGGTGAGTTCTTTGATTTTGGC	This study
Nterm_PtpA_C8S	ATGGTAGATGTAGCATTGTCTAGTCTTGGCAATATATGTCCG	This study
Nter_PtpA_flag_C8S_IF_Bam	GAAATCCCGGGGATCCATGGTAGATGTAGCATTGTCTAGTCTTGGCAATATATGTCCG	This study
Cter_PtpAflag_Xho_pDXA	ATCTATCTCGAGTTATTTATCATCATCATCTTTTGTA	This study
Nterm PtpA Bam_pGEX	TATGGATCCATGGTAGATGTAGCATTGTCTGT	This study
Cterm PtpA Hind IF_pGEX	TATCATCGATAGGCTTCTACCCCTCTTTCAAATTTGCATC	This study
MBH425	GCAATTATGAATCTTTCAATGTTGC	This study
MBH426	GCTGTTACCGAATTAAGAAAAGTTACTTACGCC	This study

^a Em^r, Erythromycin resistant; Kan^r, kanamycin resistant.

that this protein was actively retained by viable mycobacteria residing inside phagosomes (45) suggesting that the retention of coronin-1 on mycobacteria-loaded phagosomes is responsible for suppressing phagosome-lysosome formation (58). More recent work demonstrated that trimerization of coronin-1 was essential for mycobacterial survival, and that the transition from the trimer to the monomer form was regulated by serine phosphorylation (59). In the light of the latter findings one may speculate that Tyr-phosphorylation of Coro-1A might also affect the spatial distribution of this protein in *S. aureus*-infected macrophages, and that the bacterium attempts to modulate this process via PtpA. However, it is unclear yet whether Tyr-phosphorylation of Coro-1A affects the trimerization of this protein, and further work is required to understand how PtpA recognizes Coro-1A and/or other putative interactors identified by our co-immunoprecipitation studies, and whether and how they participate in the establishment of *S. aureus* survival and virulence.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strains were grown at 37 °C in LB medium supplemented with antibiotic when required. *S. aureus* isolates were plated on tryptic soy agar (BD Bioscience) supplemented with antibiotic when required, or grown in tryptic soy broth (TSB) (BD Bioscience) medium at 37 °C and 150 rpm. The Newman $\Delta ptpA$ mutant derivatives were pre-

viously constructed (34). The SA564 $\Delta ptpA$ mutant was obtained by phage transducing the *aphAIII-lox*-tagged $\Delta ptpA$ mutation from Newman $\Delta ptpA$ into the low passage clinical isolate SA564 (60).

Construction of the *S. aureus* *ptpA* *cis*-complementation strain SA564 $\Delta ptpA::ptpA$

For *cis*-complementation of the *ptpA* mutation in strain SA564 $\Delta ptpA$, a 0.8-kb fragment containing the C-terminal part of ORF RT87_RS09705 located downstream of *ptpA* (RT87_RS09695) was amplified by PCR from chromosomal DNA of *S. aureus* strain SA564 using the primer pair MBH425/MBH426 (Table 2). The resulting PCR product was digested with KpnI/EcoRI, and subsequently cloned into KpnI/EcoRI-digested vector pEC1 (61) to generate the suicide plasmid pEC1_ptpAc. *E. coli* IM08B (62)-derived plasmid pEC1_ptpAc was directly electroporated into *S. aureus* strain SA564. A SA564 derivative that integrated pEC1_ptpAc was subsequently used as a donor for transducing the *cis*-integrated pEC1_ptpAc into SA564 $\Delta ptpA$, thereby replacing the *aph*-tagged *ptpA* deletion with the WT *ptpA* genome region.

Animal studies statement

Animal experiments were approved by the local State Review Boards of Saarland and conducted according to the regulations of German veterinary law.

Murine abscess model

Preparation of the bacterial inoculum and infection of the animals were carried out as described (37), with minor modifications. Briefly, 100- μ l bacterial suspensions containing $\sim 10^7$ colony forming unit (cfu) were administered intravenously by retro-orbital injection into female, 8–10-week-old C57/BL6N mice that were anesthetized by isoflurane inhalation (5%; Baxter). Immediately after infection, mice were treated with a dose of caprofen (5 mg/kg; Pfizer), and at 4 days post-infection, mice were sacrificed, and livers were removed. The organs were weight adjusted and homogenized in PBS, and serial dilutions of the homogenates were plated on blood agar plates to enumerate the cfu.

Cloning and expression of PtpA in *D. discoideum*

For *S. aureus* PtpA-C8S overexpression in *D. discoideum*, the coding sequence of PtpA-C8S was amplified by PCR using the set of primer Nter_PtpA_flag_C8S_IF_Bam and Cter_PtpAflag_Xho_pDXA (Table 2). The amplified product was digested with BamHI and XhoI restriction enzymes and cloned into *D. discoideum* expression vector pDXA vector (63). The pDXA_PtpA-C8S-Flag plasmid was transfected in *D. discoideum* cells as described (64).

Cloning, expression, and purification of recombinant PtpA derivatives

GST-PtpA recombinant protein was obtained by cloning the *ptpA* fragment generated by PCR using *S. aureus* N315 genomic DNA as a template with the primers Nterm PtpA Bam_pGEX and Cterm PtpA Hind_pGEX (Table 2) into the BamHI-HindIII-digested pGEX vector. PtpA_C8S_His harboring cysteine to serine substitution was generated by using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies) on pETPhos_PtpA template (34) using the primer Nterm PtpA_C8S (Table 2) thus generating pETPhos_PtpA_C8S. PtpA-His and PtpA-GST derivatives were purified as previously described (34, 65).

Cloning, expression, and purification of coronin derivatives

The coding sequence of the *D. discoideum* CorA homolog was amplified by PCR from *D. discoideum* genomic DNA using the appropriate primers introducing BamHI and XhoI restriction sites, respectively (Table 2). The PCR product was digested with BamHI and XhoI, and ligated into the *D. discoideum* expression vector pDXA that was digested with the same restriction enzymes. The pDXA 3xmyc-CorA plasmid (pFL1290) was linearized with ScaI and transfected in *D. discoideum* strain DH1–10. Cells were grown at 22 °C in HL5 medium as previously described (64). Human coronin-1A (Hs-Coro-1A) (gi: 300934762, NP_001180262.1) was synthesized, codon optimized for bacterial production by GenScript, and cloned into pUC57 vector. The coding sequence was amplified by PCR from pUC57 vector, digested by NcoI-HindIII restriction enzymes, and ligated into pCDFDuet-GSTCter-up1 expression vector, thus generating pCDFDuet-Coro-1A-GST. The construct was verified by DNA sequencing. For pull-down assays, lysates of BL21 Star expressing GST-Hs-coronin-1A or GST

alone were prepared as follows. Transformed *E. coli* BL21 Star cells with human coronin-1A codon optimized were grown at 16 °C in LB medium containing 1 g/liter of glucose and 50 μ g/ml of spectinomycin and protein synthesis induced with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside overnight. Bacteria expressing GST-Hs-coronin-1A or GST alone were disrupted by sonication (Branson, digital sonifier) and centrifuged at 14,000 rpm for 25 min. Protein concentration was determined using BCA reagent (ThermoFisher Scientific, France).

Macrophage culture and infection

The murine macrophage cell line RAW 264.7 (mouse leukemic monocyte macrophage, ATCC TIB-71) was cultured in Dulbecco's modified Eagle's medium (ThermoFisher Scientific, France) supplemented with 10% fetal calf serum (ThermoFisher Scientific, France) in a humidified atmosphere at 5% CO₂ at 37 °C. For macrophage infection, *S. aureus* Newman and SA564 strains were grown to the midexponential growth phase ($A_{600} = 0.7–0.9$) in TSB medium. The bacteria were then collected by centrifugation at 10,000 rpm for 5 min and resuspended in sterile PBS. The RAW 264.7 cells (5×10^5 cells/ml, in 24-well plates) were inoculated with *S. aureus* at the m.o.i. of 20:1 (bacteria:cells) and incubated at 37 °C and 5% CO₂ for the indicated time. Subsequently, cells were washed once with PBS and the remaining extracellular bacteria were killed by incubation with gentamicin (100 μ g/ml) for 30 min. After gentamicin treatment, macrophages were rinsed twice with PBS (T0), and then further incubated in Dulbecco's modified Eagle's medium containing 5 μ g/ml of lysostaphin for 45 min and 22 h, respectively. Enumeration of intracellular bacteria was performed by lysing infected macrophages with 0.1% Triton X-100 in PBS. Macrophages lysates were serially diluted and plated on TSB agar plates that were subsequently cultivated at 37 °C for 16 h. The survival rate of bacteria was defined as follows: number of bacterial colonies at time post gentamicin/number of bacterial colonies at T0 $\times 100\%$.

Immunoprecipitation of PtpA from *D. discoideum*

For immunoprecipitations, 2×10^7 *D. discoideum* cells expressing flagged PtpA (PtpA_{-FLAG}), were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% Nonidet P-40, protease inhibitors (Roche Applied Science), and cleared by centrifugation for 15 min at 14,000 rpm. Lysate supernatants were incubated overnight at 4 °C with a monoclonal anti-FLAG antibody coated on agarose beads (Genscript). The beads were then washed five times in wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Nonidet P-40) and once in PBS. Bound proteins were migrated on SDS-PAGE and analyzed by immunoblotting or MS when required.

PtpA phosphatase activity assay

Phosphatase activity of PtpA-wt and -C8S His-tagged recombinant proteins was determined using *p*-nitrophenyl-phosphate as chromogenic substrate as previously published (34).

Immunoprecipitation of Coro-1A from infected macrophages

Murine macrophage coronin-1A (Mm_Coro-1A) was obtained from *S. aureus*-infected RAW 264.7 macrophage extracts

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by immunoprecipitation with anti-Coro-1A antibody (5 μ g, rabbit anti-coronin-1A; SAB4200078, Sigma). Briefly, the anti-Coro-1A antibody was first coupled to 50 μ l of Dynabeads-Protein G (10003D, Life Technologies) in a rotator for 10 min at room temperature and then covalently cross-linked using 5 mM BS3 (21580, Thermo Fisher Scientific) for 30 min at room temperature. Infected macrophages (m.o.i. = 20) were lysed at different time points pGt in 0.1% Triton X-100 in PBS containing a protease inhibitor mixture (complete EDTA-free protease inhibitor mixture, Roche). Antibody-coupled beads were added to these lysates and incubated in a rotator for 1 h at room temperature. Thereafter, the beads were washed twice in PBS, 0.02% Tween 20 and 3 times in phosphatase buffer (Tris-HCl 20 mM, pH 7.5, MgCl₂ 5 mM, DTT 5 mM). Immunoprecipitated samples were washed 3 times in PBS, 0.02% Tween 20 followed by elution in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting using the mouse anti-phosphotyrosine (clone 4G10, Millipore) or the rabbit anti-coronin-1A antibodies.

In vitro dephosphorylation of Mm_Coro-1A

Immunoprecipitated Mm_Coro-1A from infected macrophages was obtained as described above. To examine if phosphorylated Mm_Coro-1A is a suitable substrate of PtpA, equal amounts of beads were incubated with 2 μ g of purified PtpA. After 30 min and 1 h at 37 °C at 650 rpm, respectively, the reaction was stopped by the addition of sample buffer and proteins bound to the beads were resolved in a 4–20% SDS-PAGE. Mm_Coro-1A contents and its phosphorylation status were revealed by immunoblotting using anti-Coro-1A and anti-phosphotyrosine antibodies on the same blot.

Pulldown assays

For GST pulldown assays, GST-PtpA fusion proteins were produced as described above and bound to GSH-Sepharose 4B beads according to manufacturer's instructions (GE Healthcare). To prepare cell lysates, *D. discoideum* cells, expressing 3xmyc-Dd_Coro-A (2 \times 10⁷) were incubated 15 min in lysis buffer (20 mM HEPES buffer, pH 7.0, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100; complete protease inhibitor mixture, Roche) and centrifuged for 15 min at 14,000 rpm in a refrigerated microcentrifuge. GST beads were then incubated with cell lysates (800 μ g) overnight at 4 °C on a wheel. After three washes in lysis buffer, beads were heated at 95 °C for 10 min. Bound proteins were separated by SDS-PAGE and transferred to nitrocellulose before incubation with an anti-myc antibody (clone 9E10, Sigma). Blots were revealed with the Odyssey Western Detection System (Bio-Rad).

For His-pulldown assays, His-tagged PtpA fusion proteins were produced as described (34). Equal amounts of PtpA_{-His} Ni-NTA beads were incubated with BL21 Star lysates expressing GST-Hs Coro-1A or GST alone for 30 min at 4 °C with gentle agitation in coupling buffer (HEPES 20 mM, NaCl 100 mM, MgCl₂ 5 mM, Nonidet P-40 0.5%, glycerol 10%, imidazole 10 mM, pH 7.4). In parallel, as a control for unspecific binding, the same amount of Ni-NTA without immobilized PtpA was incubated in the same buffer with both lysates. For each Coro-1A assay, 100 μ l of the matrix with or without immobi-

lized PtpA was incubated with 5 mg of BL21 Star lysates diluted to 1 mg/ml. The matrix was collected by low speed centrifugation and then washed three times with the coupling buffer containing 50 mM imidazole. Proteins bound to the beads were recovered by the treatment with 40 μ l of Laemmli sample buffer at 95 °C for 5 min. Samples were then resolved on SDS-PAGE gel and subjected to immunoblotting with anti-GST and anti-Coro-1A antibodies, respectively.

Statistical analyses

Statistical significance was assessed using the Mann-Whitney *U* test. *p* values <0.05 were considered significant.

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