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Salmonella stimulates pro-inflammatory signaling through p21-activated kinases bypassing innate immune receptors

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Abstract

Microbial infections are most often countered by inflammatory responses initiated through the recognition of conserved microbial products by innate immune receptors resulting in pathogen expulsion [1–6]. However, inflammation can also lead to pathology. Therefore, tissues such as the intestinal epithelium, which are exposed to microbial products, are subject to stringent negative regulatory mechanisms to prevent signaling through innate immune receptors [6–11]. This presents a challenge to the enteric pathogen *Salmonella* Typhimurium, which requires intestinal inflammation to compete against the resident microbiota and to acquire the nutrients and electron acceptors that sustain its replication[12,13]. We show here that *S.* Typhimurium stimulates pro-inflammatory signaling by a unique mechanism initiated by effector proteins delivered by its type III protein secretion system. These effectors activate Cdc42 and the p21-activated kinase 1 (PAK1) leading to the recruitment of TRAF6 and TAK1 and the stimulation of NF-κB inflammatory signaling. Removal of Cdc42, PAK1, TRAF6, or TAK1 prevented the ability of *S.* Typhimurium to stimulate NF-κB signaling in cultured cells. Oral administration of a highly specific PAK inhibitor blocked *Salmonella*-induced intestinal inflammation and bacterial replication in the mouse intestine, although it resulted in a significant increase in bacterial loads in systemic tissues. Thus *S.* Typhimurium stimulates inflammatory signaling in the intestinal tract by engaging critical downstream signaling components of innate immune receptors. Furthermore, these findings illustrate the unique balance that emerges from host/pathogen co-evolution in that pathogen-initiated responses that help pathogen replication are also important to prevent pathogen spread to deeper tissues.

Keywords

Salmonella pathogenesis; innate immunity; NF-κB; p21-activated kinase; PAK1; PAK2; Cdc42; TRAF6; TAK1; bacterial pathogenesis; type III protein secretion; host-pathogen interactions; intestinal inflammation; host-pathogen co-evolution; inflammatory bowel disease

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Author contribution

H. S. & J. E. G. designed the research and analyzed data.

H. S., J. K. & M. L.-T., performed the research.

H. S. and J. E. G. wrote the manuscript with input from all the authors.

Competing interests

The authors declare no competing interests

Although inflammation is generally detrimental to bacterial pathogens, stimulation of intestinal inflammation is essential for the enteric pathogens *Salmonella* Typhimurium to be able to compete against the resident microbiota and to have access to critical nutrients that are otherwise unavailable in the un-inflamed gut [12,13]. Consequently, *S.* Typhimurium has evolved a mechanism to stimulate inflammation in the intestinal environment through the delivery of a subset of its effector proteins, SopE, SopE2, and SopB, by the type III protein secretion system encoded within its pathogenicity island 1 locus [14–16]. These effectors exert this function by redundantly activating Rho-family GTPases [17–20], which results in transcriptional responses very similar to those stimulated by agonists of receptors of the innate immune system [16]. However, the mechanisms by which the pathogen-initiated activation of these GTPase leads to a pro-inflammatory transcriptional response are not completely understood and have been the subject of some controversy [16,21]. It has been previously proposed that the activation of Rho-family GTPases by the *S.* Typhimurium effectors is sensed as a "danger associated molecular pattern" by the innate immune receptor NOD1 through unknown mechanisms [21], which through the stimulation of the associated kinase Rip2 [22,23], leads to NF-κB activation and pro-inflammatory transcriptional responses. However, previous studies have also shown that depletion of Cdc42 abolished *S.* Typhimurium stimulation of inflammatory signaling, even though Cdc42 depletion did not affect the ability of *S.* Typhimurium to activate the Rho-family GTPases Rac1 and the resulting actin cytoskeleton rearrangements that lead to bacterial internalization [16,18]. Furthermore, previous studies have also shown that *S.* Typhimurium stimulates intestinal inflammation in Rip2-defficient mice in a manner that is indistinguishable from wild type mice [16], even though these animals are completely impaired in NOD1 signaling [22,23]. To clarify these contrasting results, using CRISPR/Cas9-mediated genome editing we removed Rip2 from the cell line used in the previous studies [21] and observed that, contrary to the previous report [21] but consistent with our previous results [16], it had no effect on the ability of *S.* Typhimurium to stimulate NF-κB signaling (Fig. 1a), or to invade cultured cells (Supplementary Figure S1). As expected from previous reports [22,23], removal of Rip2 completely abolished NOD receptor signaling (Supplementary Figure S2). Similarly, NF-κB activation mediated by the transient expression of the *Salmonella* effector SopE was unaffected by the absence of Rip2 (Fig. 1b). In contrast and consistent with previous observations [16,18], CRISPR/Cas9-mediated removal of Cdc42 completely abolished SopE-stimulated NF-κB signaling (Fig. 1c) and significantly reduced NF-κB activation after wild-type *S.* Typhimurium infection (Supplementary Figure S3 and S4), although it did not affect TNFα signaling (Supplementary Figure S5). Although SopE, SopE2, and SopB activate Rho-family GTPases in a functionally redundant manner [18,24], their mechanism of action is different. The highly related effectors SopE and SopE2 are exchange factors for Rho-family GTPases and therefore activate these GTPases directly [17,25]. In contrast, SopB is a phosphoinositide phosphatase that stimulates Rho-family GTPase signaling indirectly by activating endogenous exchange factors through phosphoinositide fluxes [18]. Deletion of *sopB* from *S.* Typhimurium resulted in a significant reduction of the residual NF-κB activation observed in Cdc42-deficient cells after infection with wild-type bacteria (Fig. 1d) indicating that, in the absence of Cdc42, SopB may be able to stimulate NF-κB through more complex signaling events leading to the activation of other GTPases. Consistent with

previous observations [18], removal of Cdc42 did not affect the ability of *S.* Typhimurium to stimulate actin-mediated bacterial uptake (Fig. 1e), a phenotype dependent on other Rho-family GTPases [18]. Taken together, these results indicate that Cdc42 plays a central role in orchestrating *S.* Typhimurium-mediated inflammatory signaling. These results also indicate that contrary to previous reports [21], NOD1 signaling is not required for the ability of S. Typhimurium to stimulate inflammatory signaling in cultured cells. Furthermore, these results show that the presence of internalized *Salmonella*, or its ability to modulate actin remodeling and/or Rho-family GTPase activity *per se* is not sufficient to trigger inflammatory signaling. These observations are also consistent with previous reports indicating that removal of Rip2 [16] or critical components of the inflammasome [16,26] do not impair the ability of *S.* Typhimurium to stimulate intestinal inflammation.

To investigate the mechanisms by which *S.* Typhimurium triggers inflammatory signaling through Rho-family GTPase activation we searched for Cdc42-interacting proteins after *S.* Typhimurium infection. To ensure that the most relevant Cdc42 effectors were identified, we carried out these experiments at a time of infection when *S.* Typhimurium effectors would be expected to trigger inflammatory signaling [16,27]. We infected a cell line engineered to stably express endogenous levels of FLAG-epitope-tagged Cdc42 and interacting proteins were identified by affinity purification and LC-MS/MS analysis. We readily detected p21-activated kinase 1 (PAK1) and 2 (PAK2) as prominent Cdc42-interacting proteins in *S.* Typhimurium infected cells, although these interactors were not detected in uninfected cells (Supplementary Tables S1, S2, and Fig. S6). The interactions between Cdc42 and PAK1 or PAK2 after *Salmonella* infection were confirmed in equivalent experiments conducted in cells expressing epitope tagged versions of these proteins (Fig. 1f and Supplementary Figure S7). The p21-activated kinases (PAKs) are a family of Cdc42- and Rac-activated serine/threonine kinases involved in signal transduction leading to a variety of cellular responses [28–30]. There are 6 PAK family members, which on the bases of their structural similarity can be classified into two groups encompassing PAK1 through 3 (group I) and PAK4 through 6 (group II). Consistent with their potential involvement in the *Salmonella*-induced responses, the group I PAK kinases PAK1 and PAK2 have been previously linked to inflammatory signaling in the intestine [31,32], and we found that both PAK1 and PAK2 are prominently expressed in cultured cells known to mount a pro-inflammatory transcriptional response to *S.* Typhimurium, as well as in the mouse intestine (Supplementary Figure S8). To investigate the potential involvement of PAKs in *S.* Typhimurium stimulation of pro-inflammatory signaling, we examined the activation of NF-κB after bacterial infection in CRISPR/Cas9-generated PAK1-, PAK2-, or PAK3-deficient cell lines. We found that although removal of the different PAKs individually did not result in a significant reduction in the ability of wild-type *S.* Typhimurium to stimulate NF-κB signaling (Supplementary Figure S9), removal of PAK1 resulted in a significant reduction in NF-κB activation after SopE transient expression (Fig. 1g) or *S.* Typhimurium *ΔsopB* infection (Fig. 1h and i). PAK1-defficient cells were unaffected in their response to TNFα (Supplementary Figure S9), or in STAT3 or Erk activation after *S.* Typhimurium infection (Supplementary Figure S10). Furthermore, removal of PAK1, PAK2, or PAK3 did not affect the ability of *S.* Typhimurium to invade these cells (Supplementary Figure S11). We hypothesized that the more complex signaling stimulated by SopB [33–36] potentially involving other Rho-family GTPases (see above) [18],

may result in the engagement of multiple PAK family members. We were unable to generate cell lines defective in multiple PAK family members, which is in keeping with their redundant involvement in essential cell biological processes [28–30,37–40]. However, consistent with this hypothesis, addition of a highly specific inhibitor of group I PAK family members [41] completely abolished the ability of wild-type or *ΔsopB S.* Typhimurium to stimulate NF-κB signaling in cultured intestinal epithelial cells (Fig. 1j and 1k), or in enteroids derived from C57/BL6 mice (Fig. 1l). In contrast, addition of the inhibitor did not affect NF-κB activation after the addition of TNFα or IL1β (Supplementary Figure S12). Taken together these results indicate that Cdc42 orchestrates inflammatory signaling after *Salmonella* infection by engaging its PAK family effectors, more prominently PAK1.

Although studies have implicated PAK1 in multiple signaling cascades leading to NF-κB activation [31,32,42], the actual mechanisms by which this kinase participates in this signal transduction pathway are not understood [28–30]. To investigate the mechanisms by which PAK1 contributes to inflammatory signaling stimulated by *S.* Typhimurium we searched for interacting proteins using the approach described above for Cdc42. We engineered a cell line to stably express endogenous levels of FLAG-epitope tagged PAK1, infected them with wild-type *S.* Typhimurium, and PAK1-interacting proteins were identified by affinity purification and LC-MS/MS analysis as described above. We identified TRAF6 and mitogen-activated protein kinase kinase kinase 7 (TAK1) as prominent PAK1 interacting proteins in infected cells but not in uninfected cells (Supplementary Tables S3, S4, and Fig. S13). These interactions were verified in equivalent experiments conducted in cells expressing epitope tagged versions of these proteins (Fig. 2a). Furthermore we found that in the absence of infection, transiently expressed epitope-tagged full length PAK1 did not interact with TRAF6 (Fig. 2b), consistent with the notion that PAK1 can only engage TRAF6/TAK1 upon its Cdc42-mediated activation after bacterial infection. Consistent with this hypothesis, a constitutively active form of PAK1 lacking its auto inhibitory domain [28–30] readily interacted with TRAF6 in the absence of infection (Fig. 2b). These results indicate that *S.* Typhimurium infection and the subsequent activation of Cdc42 by the bacterial effector proteins leads to the formation of a PAK1/TRAF6/TAK1 complex.

Both TRAF6 and TAK1 are essential components of several immune receptor-initiated pro-inflammatory signaling pathways leading to NF-κB activation [43,44]. Therefore to assess their potential role in the *Salmonella*-initiated pro-inflammatory signaling, we generated TRAF6 and TAK1-deficient cell lines using CRISPR/Cas9-mediated genome editing, infected them with wild-type *S.* Typhimurium, and examined them for NF-κB activation. We found that removal of TRAF6 or TAK1 completely abolished NF-κB activation after *S.* Typhimurium infection (Fig. 2c). This phenotype was reversed by the expression of wild type TRAF6 or TAK1 in the respective defective cell lines (Fig. 2d and 2e), but not by the expression of a catalytic inactive form of TRAF6 ($TRAF6^{C70A}$) (Fig. 2d). In contrast, the ability of *S.* Typhimurium to invade cells was unaffected in TRAF6- or TAK1-deficient cells (Supplementary Figure S14). These results indicate that both the catalytic activity of TRAF6 and TAK1 are required for *S.* Typhimurium-mediated pro-inflammatory signaling. Given the known roles of TRAF6 and TAK1 in signal transduction for pathways emanating from innate immune receptors [43,44], these results provide an explanation for the strong similarities observed between the pro-inflammatory transcriptional responses resulting from *S.*

Typhimurium infection and those resulting from the stimulation of innate immune receptors [16]. In addition, these results support the notion that *Salmonella* can stimulate innate immune-like outputs in infected cells without the engagement of innate immune receptors [16].

We observed that *S.* Typhimurium infection of cultured cells resulted in the presence of a slower migrating PAK1 band in SDS-PAGE, which we showed to be due to its phosphorylation (Fig. 3a). LC-MS/MS analysis of PAK1 isolated from *S.* Typhimurium infected cells identified two phosphorylated residues, S220 and S223, the latter enriched in the slower migrating band (Fig. 3b and Supplementary Tables S5 and S6). Mutagenesis analysis further confirmed that the slower migrating band was due to the phosphorylation of S223 since infection of cells stably expressing the FLAG-tagged $PAK1^{S223A}$ mutant did not show the presence of the slower migrating band (Fig. 3c) although cells expressing $PAK1^{S220A}$ did (Fig. 3c). PAK1 is regulated by auto-inhibition of its C-terminal catalytic domain by the N-terminal auto inhibitory domain. Binding of activated Rho-family GTPases results in relieve from this auto-inhibition leading to PAK1 activation and auto-phosphorylation at multiple amino terminal residues including S220 [28–30]. However, for some phenotypes that may require persistent PAK1 activation, relieve from auto-inhibition is not sufficient for PAK1 activity, which requires an additional phosphorylation event at S223 by an exogenous kinase [45]. We found that $PAK1^{S223A}$ is unable to transduce *S.* Typhimurium-initiated NF-κB signaling (Fig. 3d) indicating that S223 phosphorylation is required for PAK1 function in the context of *Salmonella*-induced pro-inflammatory signaling. Importantly, we found that PAK1 S223 phosphorylation did not require its kinase activity (Supplementary Figure S15) and did not occur in TAK1-defficient cells (Fig. 3e and Supplementary Figure S15). These observations suggest a model in which the Cdc42-mediated activation of PAK1 (through its autophosphorylation at S220) leads to the recruitment of TRAF6 and TAK1, which subsequently results in the additional phosphorylation of PAK1 at S223 by TAK1. The mechanisms by which TAK1 is activated are not understood but it is likely the result of its TRAF6-mediated ubiquitination since we showed that the catalytic activity of TRAF6 is required *Salmonella*-induced NF-kB activation (see Fig. 2d). More experiments will be required to clarify some of the details of this signaling pathway.

We investigated the potential relevance of PAK1 signaling in *S.* Typhimurium-induced intestinal inflammation in a mouse model of infection. We first compared the ability of orally administered *S.* Typhimurium to stimulate inflammation and to replicate in the intestinal tract of PAK1-defficient [46] and C57/BL6 control mice. We found that although the production of pro-inflammatory cytokines was reduced in PAK1-deficient animals, the difference did not reach statistical significance (Supplementary Figure S16). However, the phenotype of PAK1-defficiency was more pronounced in the more simplified experimental system afforded by enteroids derived from PAK1-deficient animals, which showed much reduced NF-κB activation after S. Typhimurium infection in comparison to enteoroids derived from wild type animals (Supplementary Figure S17). We hypothesized that the weak phenotype observed in the deficient animals might be due to compensation of the absence of PAK1 by the highly related kinase PAK2, which shares several redundant activities [37–40]. In fact, we found that PAK2 was also engaged by Cdc42 during *S.* Typhimurium infection (Fig.

1f and Supplementary Tables S1, S2, and Fig. S6). Since PAK2-defficiency is embryonically lethal, to test this hypothesis we examined the effect of oral administration of a highly specific inhibitor of group I PAKs [41] on the ability of orally administered wild-type *S.* Typhimurium to stimulate inflammation and replicate in the intestinal tract. We found that oral administration of the inhibitor drastically reduced the production of pro-inflammatory cytokines (Fig. 4a) and the replication of *S.* Typhimurium in the intestinal tract (Fig. 4b), although the inhibitor itself had no effect on the replication of *S.* Typhimurium in broth culture (Supplementary Figure S18). The drastic reduction in the inflammatory response to *S.* Typhimurium was also observed in mice that had been pre-treated with streptomycin to deplete members of the intestinal microbiota (Fig. 4c) [47], except that in this case the decreased bacterial replication in the intestine was not observed (Fig. 4d). The latter is consistent with previous studies that have shown that removal of the competing intestinal microbiota by antibiotic treatment obviates the need for inflammation for *S.* Typhimurium to replicate within intestinal tissues [12]. In contrast, administration of the PAK inhibitor resulted in a significant increase in the number of *S.* Typhimurium colony forming units (c. f. u.) in systemic tissues in both, streptomycin treated (Fig. 4d) or untreated (Fig. 4b) animals. Of note, systemic administration of the PAK1 inhibitor had no effect in the production of pro-inflammatory cytokines and the replication of *S.* Typhimurium in the intestinal tract (Supplementary Figure S19). These observations indicate that, to be effective, the inhibitor must act locally on the intestinal epithelium. Furthermore, these results are consistent with previous reports indicating that, in the inflamed gut, PAK1 activation is limited to the luminal surface of the intestinal epithelium [31].

To further probe the role of PAKs in the stimulation of pro-inflammatory responses in the intestine we made use of a *S.* Typhimurium mutant lacking the type III secretion effector proteins PipA, GogA and GtgA, which dampen the intestinal inflammatory response to *Salmonella* by specifically targeting NF-κB transcription factors RelA and RelB [48]. In a NRAMP1 (SLC11A1) +/+ mouse, this mutant strain exhibits increased lethality that is not due to increased bacterial replication but to heightened production of pro-inflammatory cytokines in the gut [48]. We found that administration of the PAK inhibitor markedly reduced the production of pro-inflammatory cytokines (Fig. 4e) and the bacterial loads (Fig. 4f) in the intestinal tract, although it increased the c. f. u. in systemic tissues (Fig. 4f). More importantly, addition of the inhibitor protected orally infected animals from death due to heightened cytokine production as a consequence of infection with this *S.* Typhimurium mutant strain (Fig. 4g). Taken together these results indicate that PAKs play a central role in the coordination of the inflammatory response to *S.* Typhimurium in the intestinal tract. In addition, these results indicate that while the inflammatory response is critically important for the replication of *S.* Typhimurium within the intestine, this response is also central for the host to anatomically restrict the pathogen and prevent its access to deeper tissues.

We have described here a pathogen-specific mechanism utilized by *S.* Typhimurium to trigger intestinal inflammation without the engagement of innate immune receptors (Fig. 4h). The mechanism described here allows *Salmonella* to stimulate a response that shares great similarity with the responses stimulated by the activation of canonical innate immune receptors, while avoiding the negative regulatory mechanisms that prevent the activation of these receptors in the intestinal tract. This pathogen achieves this remarkable feat by

engaging innate immune signaling pathways downstream from the actual receptors that initiate them. These results are an example of the unique balance that emerges from the host/pathogen co-evolution in that pathogen-initiated responses that help pathogen replication are also important to prevent pathogen spread to deeper tissues. Furthermore, the mechanisms describe here could help develop anti-pathogen therapeutic strategies by targeting specific host-signaling pathways.

METHODS

Plasmids, antibodies, and reagents

All the plasmids used in this paper were generated using the Gibson assembly cloning method [49] as described previously [48]. Antibodies to IκBα (Cat. # 4814S), p-Erk (Cat. # 4370S), p-STAT3 (Cat. # 9145P), were purchased from Cell Signaling Technology, and the anti-FLAG M2 (Cat. # F1804) from Sigma (St. Louis, MO). The monoclonal antibody directed to the M45 epitope was obtained from Pat Hearing [50]. The inhibitor of PAK Group I, FRAX486 (Cat. # S7807), was purchased from Selleckchem (Houston, TX), λ phosphatase (#P0753) was from NEB (Ipswich, MA), and the Dual-Luciferase® Reporter Assay kit (Cat. # E1910) was from Promega (Madison, WI).

Bacterial strains and growth conditions

The wild-type *Salmonella enteria* serovar Typhimurium strain SL1344 [51] and the *ΔsopB* [19], *ΔinvA* [52] or Δ*pipA* Δ*gogA* Δ*gtgA* [48] mutant derivatives have been described previously. Bacteria were cultured as described [53] under conditions that stimulate the expression of the SPI-1 type III protein secretion system.

Cell culture and bacterial infections

Human intestinal epithelial Henle-407 (obtained from the Roy Curtiss III collection in 1987), and HEK293T cells (from the American Type Culture Collection) were cultured and infected with bacteria as described before [48]. Briefly, 18 hr after seeding onto tissue culture plates, cells were infected for indicated time with the indicated *S.* Typhimurium strains with the MOI indicated in the figure legends. Infected cells were treated with gentamicin (100 μg/ml) for 1 hour and in experiments involving longer infection times, the infected cells were cultured in medium with low concentration gentamicin (10 μg/ml) for the indicated times. All cell lines were routinely tested for the presence of mycoplasma by a standard PCR method. The cells were frequently checked for their morphological features, growth characteristics, and functionalities, but were not authenticated by short tandem repeat (STR) profiling.

Generation of stable cell lines expressing FLAG-epitope tagged Cdc42 or PAK1.

293T stable lines expressing FLAG-epitope-tagged CDC42 or PAK1 were generated through viral transduction using plpcx-based retroviral vector (gift from Walter Mothes Lab). Pseudotyped virus was produced by co-transfecting 1 μg plpcx-FLAG-Cdc42 or plpcx-FLAG-PAK1, 1 μg pGag/Pol and 0.5 μg pVSVG in a 3.5 cm dish of 293T cells. Cell culture supernatants were collected 48 hs after transfection and used at a dilution of 1:10 to transduce 293T cells. Transduced cells were selected with 1 μg /ml puromycin (Sigma, CAS

NO. 58–58-2) for 5–7 days to generate 293T stable lines expressing FLAG-epitope tagged CDC42 or PAK1.

Generation and infection of mouse intestinal epithelial enteroids.

The generation and culture of mouse intestinal epithelial enteroids were performed according to the protocol supplied by STEMCELL™TECHNOLOGIES (Document #28223). One hr before infection, enteroids were treated with FRAX486 (10 μM) or the DMSO vehicle alone. Then enteroids were dissociated in 200 μl DPBS by pipetting up and down at least 50 times. The suspension was centrifuged at 500 g for 5 min. The supernatant was discarded and the pellets were resuspended in 0.1 ml DMEM/F-12 containing *S.* Typhimurium (at an estimated MOI of 10) and incubate at 37 °C for 1 h. After centrifugation (500 g for 5 min), the supernatants were discarded and the pellets were resuspended with 100 μl SDS lysis buffer. Samples were subjected Western blot analysis.

Generation of CRISPR/Cas9 edited cell lines

Generation of CRISPR/Cas9 edited cell lines was carried out as described [54] following standard protocols [55] using the primers listed in Supplementary Table S7. Briefly, DNA repair templates were designed containing sequences homologous to the upstream and downstream regions of the targeting site separated by an insert containing stop codons in the three reading frames. The repair template and plasmids encoding the guide RNA (gRNA), Cas9 and a gene encoding puromycin resistance were transfected into HEK293T cells using lipofectamin 2000. The transfected cells were selected by puromycin treatment for 2 days and isolated clones were screened by PCR genotyping to identify cells with the inactivated gene of interest (Supplementary Figure S15) using the primers listed in Supplementary Table S7. In each case, several independently isolated clones were characterized for relevant phenotypes and found to have indistinguishable behavior.

Invasion Assay

The ability of *S.* Typhimurium to invade cultured cells was evaluated by the gentamicin protection assay as previously described [56].

NF-κB luciferase reporter assay

The NF-κB luciferase reporter assay has been previously described [48]. Briefly, the indicated cells were co-transfected with 20 ng of the pGl3-luc reporter plasmid encoding a NF-κB-responsive element and 20 ng of pRL-actin as internal control. Eighteen hours after transfection, cells were subjected to the indicated treatments, and the luciferase activity was measured in cell lysates.

Phosphatase treatment of cell lysates

HEK293T cells stably expressing FLAG-tagged PAK1 were seeded onto 6-well plates and ~18 hs later, infected with *S.* Typhimurium at a MOI of 10 for 40 min. Cells were then lysed in 0.1% DOC in TBS, centrifuged at 14,000 × rpm for 15 min at 4 °C, and the resulting supernatants were treated with λ phosphatase (800 units) for 2 hr at 30°C. Samples were then analyzed by SDS-PAGE and western blot with antibodies against FLAG.

Quantitative PCR

Quantitative PCR of mRNA in mouse tissues was performed as described previously [48]. Briefly, total RNA from mouse ceca were isolated using TRIzol reagent (Invitrogen) according to the manufacture's protocol, and was reversed transcribed with iScript reverse transcriptase (BIORAD). Quantitative PCR was performed using iQ SYBR Green Supermix (BIORAD) in an iCycler real time PCR machine (Bio-Rad). The Primers for Quantitative PCR have been described previously [48].

Co-immuoprecipitation Assay

HEK293T cells were seeded onto 10-cm dishes and 18–20 hs later, cells were transfected with 6 μg of plasmid DNA encoding the indicated proteins or empty vector as indicated in the figure legends. Twenty four hour after transfection, cells were either lysed or infected for 1 hr with *S.* Typhimurium at a MOI of 10 and then lysed in lysis buffer [0.5% Triton-X-100,150 mM NaCl, 20 mM Hepes (pH 7.4), 2 mM EGTA, 10 mM NaF, 2 mM DTT] containing protease inhibitors. After 15 min on ice, samples were centrifuged for 15 min at 14,000 × rpm at 4 °C. The supernatants were incubated with 20 μl of prewashed anti-FLAG M2 agarose (50% slurry, Sigma) for 4 hr at 4°C. Immune complexes were collected by centrifugation at 1500 × g for 3 min, washed three times with 1 ml of cold lysis buffer and eluted by adding 0.1 M glycine HCL pH 3.5. Samples were analyzed by SDS-PAGE (10% gel) and western blot with antibodies against M45 and FLAG.

Affinity purification and mass spectrometry analysis

Affinity purification of interacting proteins was carried out as previously described [57]. In brief, HEK293T cells stably expressing endogenous levels of FLAG-epitope-tagged Cdc42 or PAK1 were infected with wild type *S.* Typhimurium or mock infected as indicated the in figure legends. Cells were then lysed in lysis buffer [0.5% Triton-X-100,150 mM NaCl, 20 mM Hepes (pH 7.4), 2 mM EGTA, 10 mM NaF, 2 mM DTT] containing protease inhibitors for 15min in ice, and then centrifuged for 15min at 14,000 × rpm at 4 °C. The supernatants were incubated with 20 μl of prewashed anti-FLAG M2 agarose (50% slurry, Sigma) for 4 hs at 4 °C. Immune complexes were collected by centrifugation at 1,500 × g for 3 min, washed three times with 1 ml of cold lysis buffer and eluted by adding 0.1 M glycine HCL, pH 3.5. To identify interacting proteins samples were run on SDS-PAGE, lanes excised in three slices, and subjected to in gel trypsin digestion overnight, and the extracted peptides subjected to LC-MS/MS analysis as previously described [58]. To identify the phosphorylation sites of PAK1, samples were run on SDS-PAGE, and the position of the differentially phosphorylated bands was determined by conducting western blot analysis on an aliquot of the same samples. Bands were then excised and process as indicated above. LC-MS/MS data analysis was carried out using Mascot [59].

Mouse infections

The C57BL/6 SLC11A1 +/+ [48] and the C57BL/6 PAK1 −/− [46] have been previously described. Mouse infections were carried out as previously described [48] with some modification. Briefly, groups of age- and sex-matched C57BL/6 SLC11A1 +/+ mice were orally treated with the PAK Group I inhibitor FRAX486 (1 mg/each mouse) or DMSO 1 day

prior to infection. Treated mice were orally infected with the bacterial dose indicated in the figure legends contained in 100 ul of PBS. Twenty-four hours after infection, mice were treated again with the PAK Group I inhibitor FRAX486 (1 mg/each mouse) or DMSO. Mice were sacrificed at indicated times. In some experiments, mice were treated orally with Streptomycin (20 mg) one day before infection. The transcription of cytokines and the bacterial loads in the indicated tissues were performed as previously described [48]. Sample sizes were empirically determined to optimize numbers based on our previous experience with equivalent experiments. Mice were randomly assigned to the experimental groups but experimenters were not blinded to the assignment.

Ethics Statement

All animal experiments were conducted according to protocols approved by Yale University's Institutional Animal Care and Use Committee under protocol number 2016–07858. The IACUC is governed by applicable Federal and State regulations, including those of the Animal Welfare Act (AWA), Public Health Service (PHS), and the United States Department of Agriculture (USDA) and is guided by the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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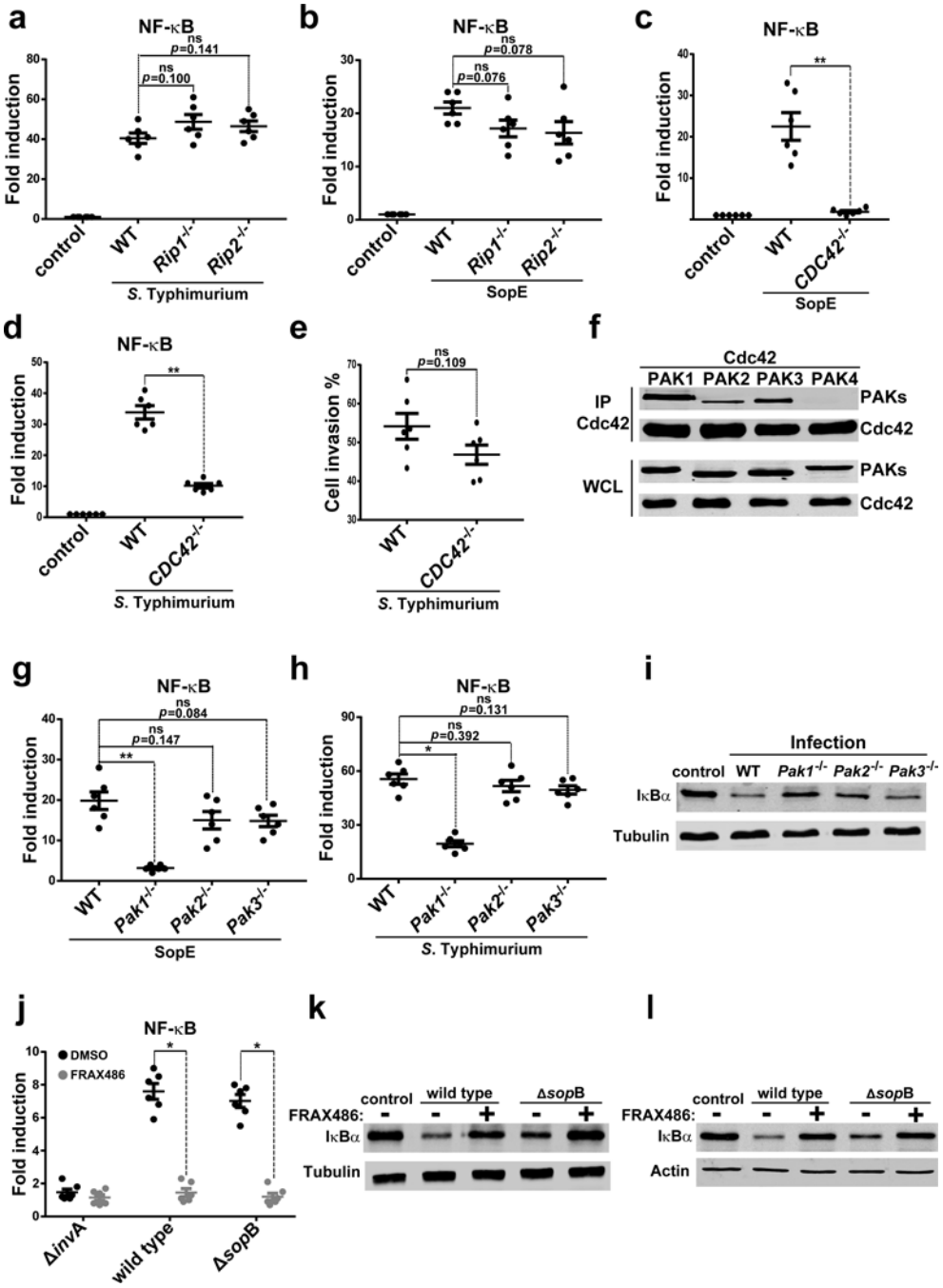

Figure 1.

Salmonella Typhimurium stimulates pro-inflammatory signaling through Cdc42 and its effector kinase PAK1. **a** and **b**, Rip1 or Rip2 are not required for *S.* Typhimurium stimulation of NF-κB signaling. HEK293T (WT), Rip1- or Rip2-defficient cells were transfected with a NF-κB luciferase reporter and subsequently infected with *S.* Typhimurium [multiplicity of infection (MOI) = 10] (**a**). Alternatively, the same cells were co-transfected with a plasmid expressing the *S.* Typhimurium effector protein SopE (**b**). Luciferase activity was measured 8 or 20 hs after bacterial infection or *sopE* DNA

transfection, respectively. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. n. s.: differences not statistically significant ($p > 0.05$ indicated in the figure, two-sided Student *t* test). **c** and **d**, NF-κB activation in CDC42-deficient cells after SopE expression or *S.* Typhimurium infection. HEK293T (WT) or CDC42-deficient cells (as indicated) were transfected with a plasmid expressing the effector protein SopE (**c**) or infected with *ΔsopB S.* Typhimurium (MOI = 10) (**d**) and the NF-κB activation was measured with a luciferase reporter as indicated above. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. Asterisks indicate that the values were statistically different from the wild-type control (** $p<0.01$, two-sided Student *t* test). **e**, *S.* Typhimurium invasion of CDC42-deficient cells. HEK293T (WT) (as controls) or CDC42-deficient cells were infected with wild-type *S.* Typhimurium (MOI = 10) and the levels of internalized bacteria were determined by the gentamicin protection assay. Results represent the % of the inoculum that survived the gentamicin treatment due to bacterial internalization and are the mean ± SD of three independent determinations. n. s.: differences not statistically significant ($p > 0.05$ indicated in the figure, two-sided Student *t* test). **f**, Interaction of Cdc42 with PAK kinases after *S.* Typhimurium infection. HEK293T cells were transiently co-transfected with plasmids encoding FLAG-epitope-tagged Cdc42 along with plasmids encoding M45-tagged PAK1, PAK2, PAK3 or PAK4. Eighteen hours after transfection, cells were infected with *S.* Typhimurium (MOI = 30) for 1 hr and cell lysates were analyzed by immunoprecipitation with anti-FLAG and western immunoblotting with anti-M45 and anti FLAG antibodies. IP: immunoprecipitates; WCL: whole cell lysates. (**g-i**) NF-κB activation in PAK-deficient cells after SopE expression or *S.* Typhimurium infection. HEK293T (WT), PAK-1, PAK2, or PAK-3-deficient cells (as indicated) were co-transfected with a NF-κB luciferase reporter and a plasmid expressing the *S.* Typhimurium effector protein SopE (**g**), or infected with *ΔsopB S.* Typhimurium (MOI = 10) (**h**). The luciferase activity was measured 20 and 8 hs after *sopE* DNA transfection or bacterial infection, respectively. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. Asterisks indicate that the values were statistically different from the wild-type control (** $p<0.01$; * $p<0.05$,); n. s.: differences not statistically significant, ($p > 0.05$ indicated in the figure), two-sided Student *t* test. Alternatively, the levels of IκBα in *S.* Typhimurium-infected cells were examined by immunoblot with anti IκBα and anti tubulin (as loading control) antibodies (**i**). **j-l,** Effect of a Group I PAKs inhibitor on NF-κB activation after *S.* Typhimurium infection. Cultured intestinal epithelial Henle-407 cells were transfected with a NF-κB luciferase reporter, treated with the PAK inhibitor FRAX486 (10 μM) or its solvent DMSO for 1 hour, subsequently infected (MOI = 10) with wild type, Δ*invA* (a type III secretion defective mutant), or Δ*sop*B *S.* Typhimurium strains, and the luciferase activity was measured 8 hs after bacterial infection. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. Asterisks indicate that the values were statistically different from the wild-type control (* $p<0.05$, two-sided Student *t* test) (**j**). Alternatively, Henle-407 cells (**k**) or enteroids derived from wild type C57/BL6 mice (**l**) treated with the PAK inhibitor FRAX486 (10 μM) or its solvent DMSO for 1 hour, were infected with wild type S. typhimurium and the levels of IκBα in were examined by immunoblot with anti IκBα and anti actin or anti

tubulin (as loading controls) antibodies. Experiments shown in **f**, **i**, **k,** and **l** were independently repeated at least three times with equivalent results.

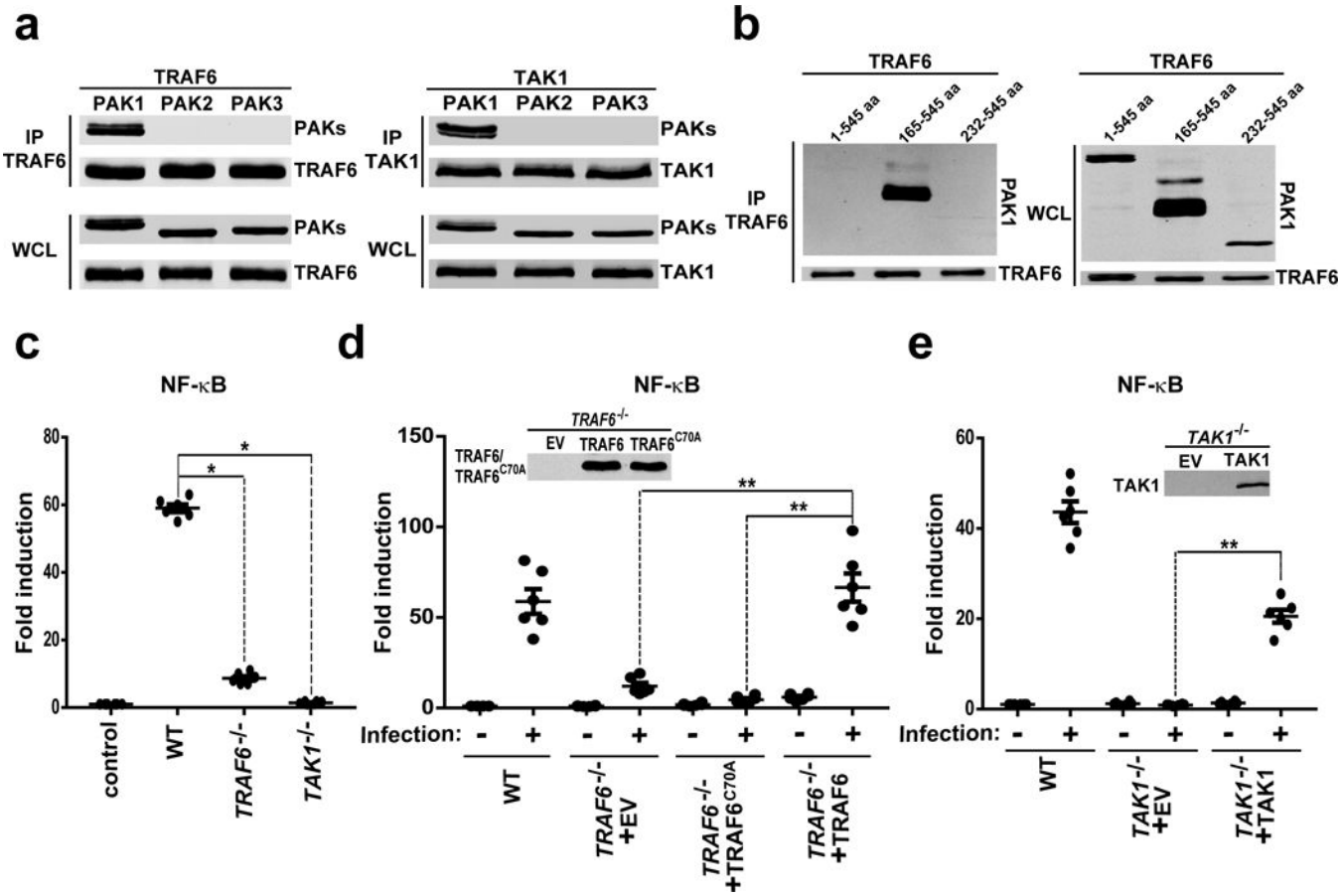

Figure 2.

PAK1-TRAF6-TAK1 mediate *Salmonella* Typhimurium pro-inflammatory signaling downstream of CDC42. **a,** PAK1 forms a complex with TRAF6 and TAK1 after *S.* Typhimurium infection. HEK293T cells were transiently co-transfected with plasmids expressing FLAG-epitope-tagged TRAF6 or TAK1 along with plasmids encoding M45-tagged PAK1, PAK2, or PAK3. Eighteen hours after transfection, cells were infected with *S.* Typhimurium for 1 hr (MOI = 30) and cell lysates were analyzed by immunoprecipitation with anti-FLAG and western immunoblotting with anti-M45 and anti FLAG antibodies. IP: immunoprecipitates; WCL: whole cell lysates. **b**, Interaction between TRAF6 and different PAK1 deletion mutants. HEK293T cells were transiently co-transfected with plasmids expressing FLAG-epitope-tagged TRAF6 along with plasmids encoding M45-tagged full-length (1–545) PAK1 or the indicated deletions and 20 hs after transfection cell lysates were analyzed by immunoprecipitation with anti-FLAG and western immunoblotting with anti-M45 and anti FLAG antibodies. IP: immunoprecipitates; WCL: whole cell lysates**. c – e,** NF-κB activation in TRAF6- or TAK1-deficient cells after *S.* Typhimurium infection. HEK293T (WT), TRAF6-, or TAK1-deficient cells (as indicated) were transfected with a NF-κB luciferase reporter and subsequently infected with *S.* Typhimurium (MOI = 10) (**c**). Alternatively, TRAF6-, or TAK1-deficient cells stably expressing endogenous levels of the wild type alleles of TRAF6, its catalytic mutant $TRAF6^{C70A}$, or TAK1 (as indicated) or carrying the empty vector (EV), were infected with *S.* Typhimurium in the same manner (**d**

and **e**). The luciferase activity was measured 8 hs after bacterial infection. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. Asterisks indicate that the values were statistically different from the wild-type control (** $p<0.01$, * $p<0.05$, two-sided Student t test). Experiments shown in **a**, and **b** were independently repeated at least three times with equivalent results.

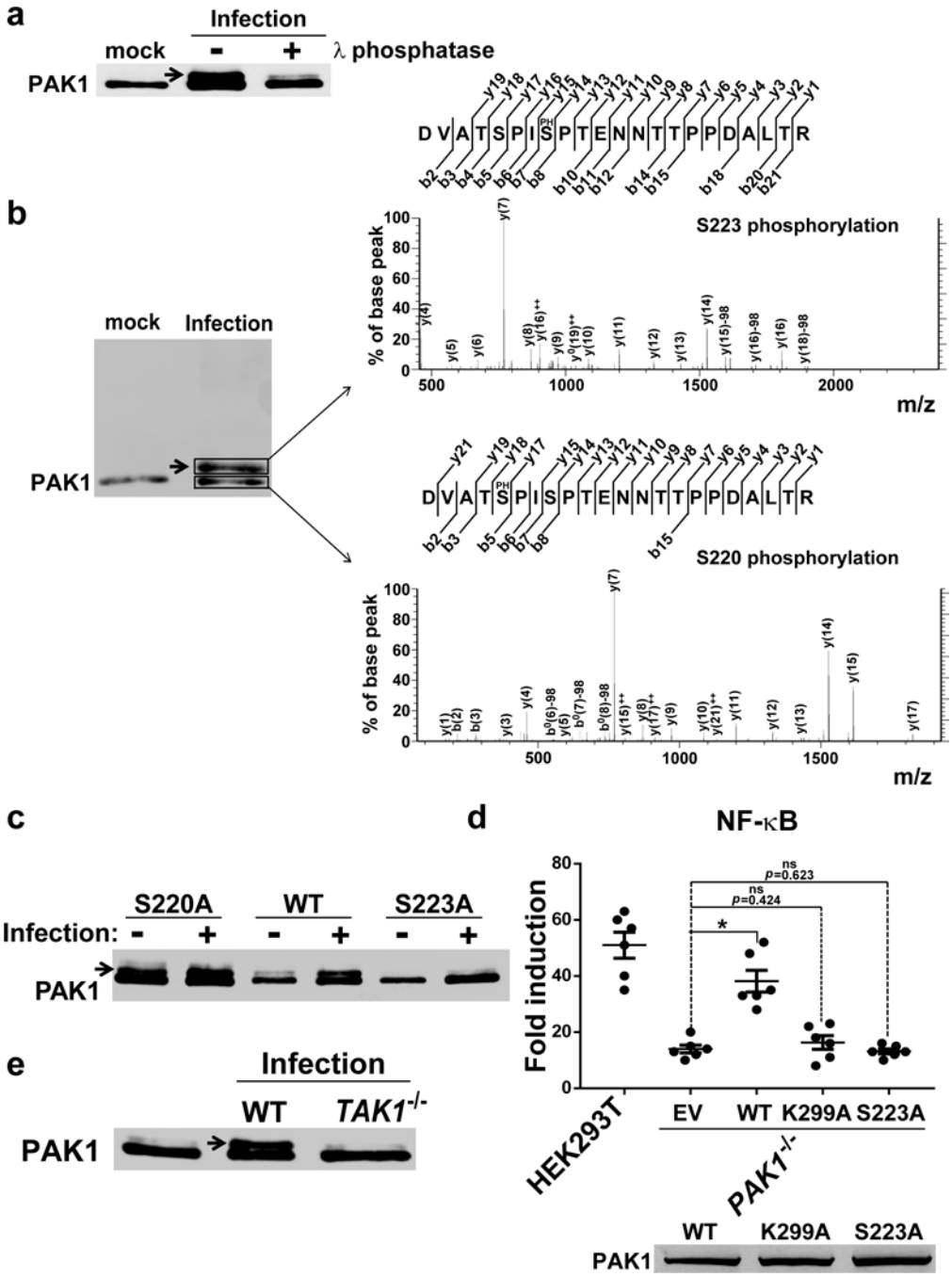

Figure 3.

Salmonella Typhimurium stimulates TAK1-dependent PAK1 phosphorylation, which is essential for pro-inflammatory signaling. **a**, PAK1 is phosphorylated upon *S.* Typhimurium infection. HEK293T cells stably expressing FLAG-tagged PAK1 were infected with wild-type *S.* Typhimurium for 1 hr (MOI = 30) and cell lysates were analyzed by immunoblot with an anti FLAG antibody before and after λ phosphatase treatment. **b**, Analysis of phosphorylation sites in PAK1 after *S.* Typhimurium infection. PAK1 was isolated from HEK293T cells stably expressing FLAG-tagged PAK1 by immunoprecipitation before and

after wild-type *S.* Typhimurium infection (MOI = 30) and separated by SDS-PAGE. Bands corresponding to the slower (phosphorylated) and normal migrating species were subjected to LC-MS/MS analysis to map the phosphorylation sites as indicated in Materials and Methods. **c** and **d**, PAK1 S223 phosphorylation is required for *S.* Typhimurium pro-inflammatory signaling. HEK293T cells stably expressing FLAG-epitope tagged PAK1 (WT), or its phosphorylation-site mutants ($PAK1^{S220A}$ or $PAK1^{S223A}$) were infected with S. Typhimurium (MOI = 30) and whole cell lysates were analyzed by immunoblotting (**c**). Alternatively, HEK293T cells or PAK1-deficient cells stably expressing wild type PAK1 (WT), its kinase inactive ($PAK1^{K299A}$), or phosphorylation-site mutant ($PAK1^{S223A}$) were infected with *S.* Typhimurium (MOI = 10) and NF-κB activation was measured 8 hs after bacterial infection with a luciferase reporter as indicated above. Values represent fold induction over control and are the mean ± standard deviation of three independent measurements. Asterisks indicate that the values were statistically different from the wild-type control (* $p<0.05$); n. s.: differences not statistically significant ($p > 0.05$ indicated in the figure), two-sided Student t test. (**d**). e, PAK1 S223 phosphorylation requires TAK1. HEK293T or TAK1-deficient cells stably expressing FLAG-tagged PAK1 were infected with *S.* Typhimurium (MOI = 30) and analyzed by immunoblot with an anti-FLAG antibody. Arrows indicate the position of phosphorylated PAK1 species. Experiments shown in **a**, **b**, **c** and **e** were repeated at least three times with equivalent results.

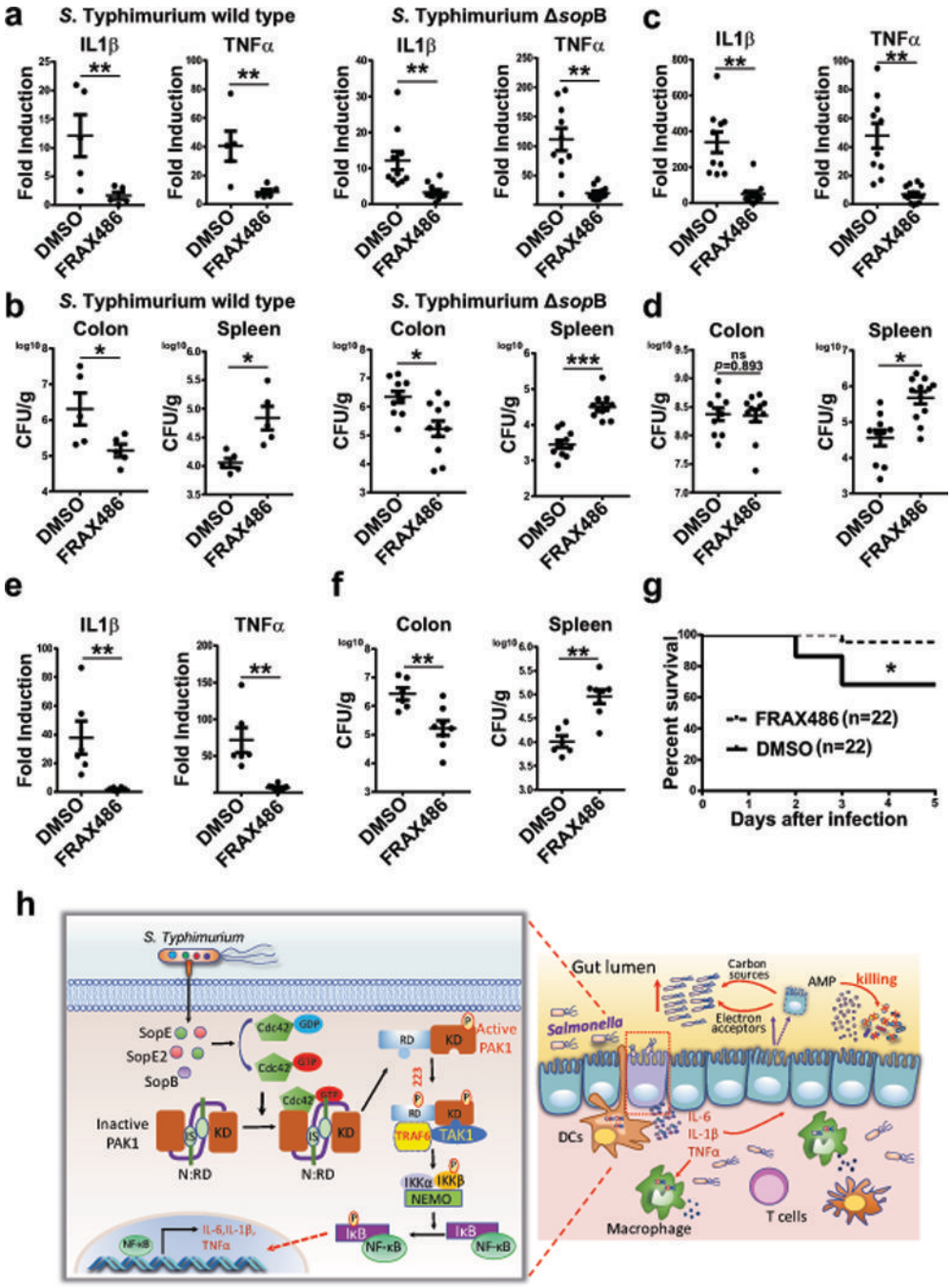

Figure 4.

PAK-mediated pro-inflammatory signaling is required for *S.* Typhimurium replication within the intestine and for the host response that limits systemic infection. **a – d**, C57/BL6 nramp+/+ mice were orally treated with DMSO or the group I PAK inhibitor FRX486 and subsequently orally infected with wild type or Δ*sop*B S. Typhimurium (as indicated) (**a** and **b**). Alternatively, mice were orally administered 20 mg of streptomycin (to deplete the intestinal microbiota) 24 hs prior to infection (**c** and **d**). Four days after infection, the relative levels of pro-inflammatory cytokine mRNAs in the intestine were measured by quantitative

real time PCR (**a** and **c**.) Each circle in **a** and **c** represent the relative levels of the indicated cytokines normalized to the levels of GAPDH; the mean ± SEM of all the measurements are shown. Each circle in **b** and **d** represent the bacterial loads the indicated tissues of individual animals; the mean ± SEM of all the measurements are shown. The results are the combination of at least two independent experiments. Asterisks indicate that the values were statistically different from the DMSO treated controls (* $p<0.05$; ** $p<0.01$; *** $p<0.001$, two-sided Student *t* test). (**e-g**) A PAK inhibitor protects animals from intestinal cytokine-mediated death after *Salmonella* infection. C57BL/6 nramp+/+ mice were orally treated with DMSO or the group I PAK inhibitor FRX486 and subsequently orally infected with the Δ*pipA* Δ*gogA* Δ*gtgA* S. Typhimurium, a mutant that stimulates an augmented intestinal inflammatory response. Four days after infection, the relative levels of pro-inflammatory cytokine mRNAs in the intestine were measured by quantitative real time PCR (**e**). Each circle in **e** represents the relative levels of the indicated cytokines normalized to the levels of GAPDH; the mean ± SEM of all the measurements are shown. Each circle in **f** represents the bacterial loads the indicated tissues of individual animals; the mean ± SEM of all the measurements are shown. The results are the combination of at least two independent experiments. Asterisks indicate that the values were statistically different from the DMSO treated controls (** $p<0.01$, two-sided Student *t* test). Alternatively, the survival of animals at the indicated times was scored (**g**). The *p* value of the difference between the survival of animals treated with wild DMSO or FRAX486 was determined by the log-rank test (* $p<0.05$). The results are the combination of two independent experiments. (**h**) Model for the interaction of *Salmonella* with the intestinal epithelium. Through its type III secretion system encoded within its pathogenicity island 1, *Salmonella* delivers the effector proteins SopE, SopE2, and SopB, which in a functionally redundant manner activate Cdc42. Activation of Cdc42 leads to the formation of a PAK1/TRAF6/TAK1 complex, the phosphorylation of PAK1 at Ser223, and the subsequent activation of NF-κB and production of pro-inflammatory cytokines. The ensuing intestinal inflammation allows *Salmonella* to out-compete the resident microbiota and replicate within the lumen of the intestinal tract. At the same time, the inflammatory response controls the spread of *Salmonella* to deeper tissues.