β-arrestin 2 quenches TLR signaling to facilitate the immune evasion of EPEC

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ABSTRACT
The protein translocated intimin receptor (Tir) from enteropathogenic Escherichia coli shares sequence similarity with the host cellular immunoreceptor tyrosine-based inhibition motifs (ITIMs). The ITIMs of Tir are required for Tir-mediated immune inhibition and evasion of host immune responses. However, the underlying molecular mechanism by which Tir regulates immune inhibition remains unclear. Here we demonstrated that β-arrestin 2, which is involved in the G-protein-coupled receptor (GPCR) signal pathway, interacted with Tir in an ITIM-dependent manner. For the molecular mechanism, we found that β-arrestin 2 enhanced the recruitment of SHP-1 to Tir. The recruited SHP-1 inhibited K63-linked ubiquitination of TRAF6 by dephosphorylating TRAF6 at Tyr288, and inhibited K63-linked ubiquitination and phosphorylation of TAK1 by dephosphorylating TAK1 at Tyr206, which cut off the downstream signal transduction and subsequent cytokine production. Moreover, the inhibitory effect of Tir on immune responses was diminished in β-arrestin 2-deficient mice and macrophages. These findings suggest that β-arrestin 2 is a key regulator in Tir-mediated immune evasion, which could serve as a new therapeutic target for bacterial infectious diseases.

Introduction
Enteropathogenic Escherichia coli (EPEC) belongs to a group of pathogenic organisms that adhere to the intestinal epithelial cell surface to form an attaching and effacing lesion.1,2 It continues to be a significant cause of infantile diarrhea in developing countries and contributes to high morbidity and mortality.3-5 Translocated intimin receptor (Tir) is one of the most important virulence factors that is essential for disease development.6,7 Tir is injected into host cells by a type III secretion system (T3SS) and spans the host cell membrane with its amino and carboxyl termini in the host cytoplasm. The central extracellular domain of Tir is engaged by the bacterial surface ligand intimin, which mediates intimate adhesion of the bacteria to host cells.8 Intimin-dependent clustering of Tir triggers the host protein N-WASP to form the Arp2/3 complex, leading to host cytoskeleton rearrangement to form pedestals, which promotes bacterial colonization and infection.9,10 Besides its role in actin rearrangement, Tir shares sequence similarity with cellular immunoreceptor tyrosine-based inhibition motifs (ITIMs), which play an inhibitory role and promote immune evasion of EPEC.11 However, the underlying molecular mechanism by which Tir regulates immune inhibition remains elusive.

Toll-like receptor 4 (TLR4) plays a pivotal role in early host defense against gram-negative bacteria. Upon ligand stimulation, TLR4 dimerizes and undergoes conformational change required for association of the adaptor protein myeloid differentiation
primary response protein 88 (MyD88), which in turn recruits IL-1 R-associated kinase 4 (IRAK4) and IL-1 R-associated kinase 1 (IRAK1). Tumor necrosis factor receptor-associated factor 6 (TRAF6) is then recruited to the signal complex via interaction with phosphorylated IRAK1, which subsequently associates with the ubiquitin ligases ubiquitin-conjugating enzyme 13 and ubiquitin-conjugating enzyme E2 variant. These results suggest that β-arrestin 2 plays roles in host and microbe interaction is unclear.

For example, β-arrestin 2 interacts with TRAF6 to negatively regulate TLR signaling in innate immunity, inhibits insulin resistance by regulating Src and Akt signaling, and interacts with KIR2DL1 in NK cells to facilitate inhibitory signaling. These results suggest that β-arrestin 2 functions in various signaling pathways by interacting with different molecules. However, whether β-arrestin 2 plays important role in regulating the immune evasion process during enteric bacterial infection.

Results

β-arrestin 2 mediates inhibition of the cytokine production by Tir

Bacterial infection triggers the activation of TLR signaling pathways and induces cytokines production, which is essential in activating immune responses. However, many bacterial pathogens have evolved various strategies to evade the host immune responses. For example, Tir protein is injected into immune cells by EPEC and inhibits pro-inflammatory cytokines production by recruiting the tyrosine phosphatase SHP-1. To determine whether β-arrestin 2 plays a role in EPEC infection, we challenged primary peritoneal macrophages from WT and β-arrestin 2-deficient mice with wild-type EPEC (JPN15 strain) and a mutant from which the gene encoding Tir was deleted (EPEC Δtir) to assess the ability of β-arrestin 2 on Tir-mediated inhibition of cytokine production. We obtained RNA from primary peritoneal macrophages infected for 6 h and analyzed the expression of genes encoding proinflammatory cytokines TNF-α, IL-6, IL-12, and IL-1β by quantitative real-time PCR. The results showed the β-arrestin 2-deficient cells infected with EPEC exhibited increased production of cytokines compared with wild-type cells infected with EPEC, which was similar to the EPEC Δtir infected groups with or without β-arrestin 2 (Figure 1(a)), indicating...
that the deletion of β-arrestin 2 significantly reduced the inhibitory effect of Tir on the expression of proinflammatory cytokines. Next, we transfected RAW264.7 cells with negative control small-interfering RNA (siRNA) and β-arrestin 2-specific siRNA and assessed the expression of β-arrestin 2 by immunoblot analysis. We confirmed that β-arrestin 2-specific siRNA significantly decreased the expression of β-arrestin 2 (Figure S1A). Similarly, inhibition of the expression of β-arrestin 2 by siRNA in RAW264.7 resulted in much higher expression of TNFA, IL6, IL12, and IL1B in response to infection with EPEC than that in RAW264.7 cells transfected with negative control siRNA. Conversely, the expression of these cytokines had no difference in RAW264.7 cells transfected with negative control siRNA or β-arrestin 2 specific siRNA and infected with EPEC Δtir (Figure S1B). Next, we analyzed the phosphorylation of signal molecules in the MAP kinase and NF-κB signaling pathway in mouse primary peritoneal macrophages and RAW264.7 cells. As expected, phosphorylation of Erk, Jnk, p38, p65, and IKKa/β was apparently inhibited in wild-type cells compared with that in β-arrestin 2-deficient primary peritoneal macrophages or RAW264.7 cells transfected with β-arrestin 2-specific siRNA and infected with EPEC. However, the inhibitory effect disappeared upon infection with EPEC Δtir, irrespective of the presence of β-arrestin 2 (Figure 1(b) and Figure S1 C). Consistent with inhibition of activation of Erk, Jnk, p38, p65, and IKKa/β, the amount of TNF-α, IL-6, IL-12, and IL-1β (cleaved active form) in the medium of mouse primary peritoneal macrophages infected with EPEC is less than that in β-arrestin 2 deficient macrophages (Figure 1(c)). All of these findings suggest that Tir inhibits the production of cytokines in a β-arrestin 2-dependent manner in immune cells.

Figure 1. Inhibition of cytokine production by Tir is mediated by β-arrestin 2. (a) Quantitative RT-PCR analysis of TNFA, IL6, IL12, and IL1B mRNA in wild-type or β-arrestin 2-deficient macrophages infected for 6 h with EPEC or EPEC Δtir. (b) Immunoblot analysis of lysates of mouse primary macrophages infected for 0–4 h (above lanes) with EPEC or EPEC Δtir. Densitometry quantification of each blot was measured with ImageJ Software. Numbers under the blots represent the ratio that the densitometry value of each blot was compared with the value of reference blots. (c) ELISA analysis of cytokine production in supernatants of mouse primary macrophages infected for 4 h with EPEC or EPEC Δtir. Arrb2, β-arrestin 2. Arrb2+/−, wild-type mice. Arrb2−/−, β-arrestin 2-deficient mice. Data are representative of at least three independent experiments. Error bars show mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 (two-way ANOVA).
Interaction of β-arrestin 2 with Tir

To confirm the interaction of β-arrestin 2 and Tir, we performed co-immunoprecipitation (Co-IP) assay. Using lysates of human embryonic kidney cells (HEK293 T cells) expressing Flag-tagged Tir and HA-tagged β-arrestin 2, we found that β-arrestin 2 immunoprecipitated together with Tir in the presence of the tyrosine phosphatase inhibitor pervanadate (Figure 2(a,b)), suggesting that their interaction is dependent on tyrosine phosphorylation. We further examined the interaction between Tir and endogenous β-arrestin 2 from mouse primary peritoneal macrophages infected with EPEC (Δtir + HA-tir) (a JPN15 Tir strain transformed with HA-tagged Tir encoded by the vector pK184). In this model system, we found that endogenous β-arrestin 2 was immunoprecipitated by Tir (Figure 2(c)). Tir could also be pulled down by β-arrestin 2 in HeLa (human cervical cancer cells) cells over-expressing Flag-tagged β-arrestin 2 and infected with EPEC (Δtir + HA-tir) (Figure S2). Next, we used HeLa cells engineered to express Flag-tagged β-arrestin 2 and infected with EPEC Δtir or EPEC (Δtir + HA-tir) to examine the intracellular localization of Tir and β-arrestin 2. HA-tagged Tir and Flag-tagged β-arrestin 2 localized together, as assessed by immunofluorescence confocal microscopy (Figure 2(d)). To determine whether they interact directly, we used the E. coli TBK1 strain with a plasmid-encoded inducible gene encoding tyrosine kinase to express and purify tyrosine-phosphorylated S-transferase (GST) fusion Tir to incubate with purified recombinant histidine-β-arrestin 2 (His-β-arrestin 2). The results showed that β-arrestin 2 directly bound with GST-Tir (Figure 2(e)).

The carboxy terminus of Tir contains two ITIMs. As the interaction between β-arrestin 2 and Tir occurs only in the presence of pervanadate (Figure 2(a,b)), we suppose that their interaction requires tyrosine phosphorylation of ITIM. We generated Tir mutants in which either one or both tyrosine in ITIMs were replaced with phenylalanine to determine whether phosphorylation of Tir is necessary for its interaction with β-arrestin 2 (Figure 2(f)). We found that the substitution of Tyr483 or Tyr511 resulted in decreased interacting ability and that the substitution of both Tyr483 and Tyr 511 led to even less interaction (Figure 2(g)). We then expressed GST-tagged Tir and its single- or double-point mutants in the E. coli TBK1 strain, which allowed tyrosine phosphorylation of the Tir protein, to determine whether the phosphorylation of ITIM was required for direct binding (Figure 2(h)). The result showed that the tyrosine phosphorylation of Tir was decreased in the mutants (Figure 2(i)). Using these purified recombinant proteins in an in vitro GST precipitation assay, we found that only Tir with phosphorylated tyrosine in ITIMs associated with purified recombinant His-tagged β-arrestin 2 (Figure 2(j)), endogenous β-arrestin 2 from mouse primary peritoneal macrophages (Figure 2(k)) or RAW264.7 cells (Figure 2(l)), whereas substitution of tyrosine residues in ITIMs eliminated this association. These results indicated that direct interaction between Tir and β-arrestin 2 is dependent on tyrosine phosphorylation of ITIM. There are six tyrosine residues in Tir amino acid sequence. In order to determine which tyrosine kinase phosphorylated the tyrosines in ITIM, we constructed a Tir mutant in which the other four tyrosine residues were mutated to phenylalanine except for the two in ITIMs. The results showed that Tir ITIMs could be phosphorylated by host Frk, Lyn, Src, and Blk (Figure 2(m)).

β-arrestin 2 facilitates the interaction of SHP-1 and Tir

ITIM-phosphorylated Tir recruits SHP-1 to inhibit the activation of the TLR pathway. To determine the role of β-arrestin 2 in this complex, we co-expressed β-arrestin 2 with Tir and SHP-1 in HEK293 T cells. The results showed that overexpression of β-arrestin 2 enhanced the association of Tir and SHP-1 in a dose-dependent manner (Figure 3(a)). Conversely, β-arrestin 2 deficiency in mouse primary peritoneal macrophages or knockdown of β-arrestin 2 by siRNA in RAW 264.7 cells reduced endogenous interaction between SHP-1 and Tir (Figure 3(b,c)). SHP-2 is another important tyrosine phosphatase and usually has similar roles as SHP-1. Using a Co-IP assay, we found that β-arrestin 2 significantly promoted the binding of SHP-2 to Tir (Figure S3A), while deficiency of β-arrestin 2 reduced the endogenous interaction between SHP-2 and Tir in mouse primary peritoneal macrophages (Figure S3B).
β-arrestin 2 promotes the inhibitory effect of SHP-1 on TRAF6

The activation of TRAF6 depends on its autoubiquitination. Here we found that β-arrestin 2 promoted the recruitment of SHP-1 to TRAF6 in a dose-dependent manner (Figure 4(a)). Furthermore, the interaction of β-arrestin 2 with Tir promoted the binding of SHP-1 and TRAF6 (Figure 4(b)). K48-
linked ubiquitination usually mediates the degradation of proteins, whereas K63-linked ubiquitin chains act as scaffolds for assembling protein kinases and induce their activation. Here we observed that SHP-1 inhibited K63-linked ubiquitination of TRAF6, but had no effect on K48-linked ubiquitination (Figure 4(c,d)). To further determine the role of β-arrestin 2 in Tir-mediated inhibition of TRAF6 ubiquitination, we isolated peritoneal macrophages from wild-type and β-arrestin 2-deficient mice and infected them with EPEC or EPEC Δtir. β-arrestin 2 deficiency significantly increased total and K63-linked TRAF6 ubiquitination but had no effect on K48-linked ubiquitination in mouse primary macrophages infected with EPEC (Figure 4(e)). Conversely, β-arrestin 2 deficiency had no significant effect on TRAF6 ubiquitination in cells infected with EPEC Δtir (Figure 4(e)). By the dual-luciferase reporter assay, we found that SHP-1 inhibited the activation of NF-κB and MAPK signaling pathways triggered by TRAF6 (Figure 4(f,g)). These results show that Tir-induced inhibition of K63-linked TRAF6 ubiquitination through SHP-1 is mediated by β-arrestin 2.

Here we questioned how protein tyrosine phosphatase SHP-1 could inhibit K63-linked ubiquitination of TRAF6. We reasoned that SHP-1 might inhibit the tyrosine phosphorylation of TRAF6 to inhibit its ubiquitination. We mutated all the 14 tyrosine in TRAF6 to phenylalanine, and then detected the change of K63-linked ubiquitination of the mutant. The result showed that mutations at 68, 217, 231, 288, 353, 381, 387, 406, 413, and 418 residues significantly decreased the K63-linked ubiquitination of TRAF6 (Figure S4). When the above mutants were co-transfected with SHP-1, all of them showed even lower K63-linked ubiquitination modification except for Tyr288 (Figure 4(h)), suggesting that Tyr288 is the target of SHP-1. It indicated that SHP-1 inhibits K63-linked ubiquitination of TRAF6 by dephosphorylating Tyr288.

In addition, we found that β-arrestin 2 inhibited the interaction of SHP-2 and TRAF6 (Figure S5A). SHP-2 increased total ubiquitination of TRAF6 to a slight extent and K63-linked ubiquitination to a significant extent but inhibited K48-linked ubiquitination (Figure S5B-D). Overall, β-arrestin 2 mediated the inhibitory effect of Tir on TLR signaling via SHP-2 in a different manner compared with SHP-1.

**β-arrestin 2 promotes the inhibitory effect of SHP-1 on TAK1**

The interaction of TAK1 and TAB1 is essential for the activation of TAK1. We performed Co-IP experiment and found that SHP-1 interacted with TAK1, which was further promoted by β-arrestin 2 (Figure 5(a)). Meanwhile, SHP-1 reduced the interaction of TAB1 and TAK1 (Figure 5(b)). Conversely, β-arrestin 2 deficiency enhanced the endogenous interaction of TAK1 and TAB1 in mouse primary peritoneal macrophages infected with EPEC, while the interaction stayed at a high level when cells were infected with EPEC Δtir.
Besides, overexpression of SHP-1 in HEK293 T cells resulted in less phosphorylation of TAK1 at Ser187 residue, which is the most important phosphorylation site for the activation of TAK1 (Figure 5(d)).

Figure 5(c).)

Although β-arrestin 2 promoted the interaction of SHP-2 and TAK1 (Figure S6A), SHP-2 had no effect on TAK1 phosphorylation (Figure S6B). To determine whether β-arrestin 2 was involved in the inhibitory effect of Tir on the activation of TAK1, we infected mouse primary macrophages isolated from wild-type or β-arrestin 2-deficient mice with EPEC or EPEC Δtir. WT, Arrb2+/−, KO, Arrb2−/−. (f,g) Luciferase assay of HEK293 T cells transfected for 18 h with indicated plasmids. (h) Immunoassay of lysates of HEK293 T cells expressing TRAF6 or TRAF6 mutants in which tyrosines were mutated to phenylalanines. WCL, whole-cell lysate. Data are representative of at least three independent experiments. Error bars show mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 (two-way ANOVA).
higher amounts of phosphorylation of TAK1 after infection with EPEC Δtir (Figure 5(e)). We obtained similar results in RAW264.7 cells in which the expression of β-arrestin 2 had been knocked down by RNA-mediated interference (Figure S7A).

K63-linked ubiquitination of TAK1 leads to the formation of the TRAF6–TAK1–MAP kinase kinase kinase 3 (MEKK3) complex, which contributes to sustained activation of NF-κB. Using lysates of HEK293 T cells expressing various proteins, we observed that phosphorylation of TAK1 was increased in cells infected with EPEC Δtir for 4 h compared to those infected with EPEC (Figure 5(e)). This increase was not observed in cells expressing β-arrestin 2 (Figure 5(f)). Immunoblot analysis of lysates of mouse primary macrophages cells infected with EPEC or EPEC Δtir for 0–4 h showed increased phosphorylation of TAK1 in cells expressing β-arrestin 2 (Figure 5(g)). We then performed an immunoblot assay of lysates of HEK293 T cells expressing various proteins (above lanes) to confirm these findings (Figure 5(h)).
and a plasmid-expressing ubiquitin, K63-linked ubiquitin, or K48-linked ubiquitin, we found that SHP-1 inhibited total or K63-linked ubiquitination, but had no effect on K48-linked ubiquitination of TAK1 (Figure 5(f,g), Figure S7 C). Using the dual-luciferase reporter assay system, we found that SHP-1 inhibits the activation of NF-κB and MAPK signaling pathways triggered by TAK1/ TAB1 (Figure 5(h,i)).

Similar to TRAF6, we constructed 15 mutants of TAK1 in which each tyrosine was mutated to phenylalanine. The phosphorylation of TAK1 was inhibited when 106, 206, or 506 residues were mutated (Figure S7B). Over-expression of SHP-1 did not further decrease the phosphorylation of TAK1 (Y206 F) (Figure 5(j)), indicating that SHP-1 might inhibit the phosphorylation of TAK1 at Tyr206 to suppress its phosphorylation of TAK1 at Ser187. The K63-linked ubiquitination of TAK1 decreased when tyrosine in 33, 113, 20, 300, 506, 557, and 558 residues were mutated to phenylalanine (Figure S7D), while over-expression of SHP-1 did not further decrease K63-linked ubiquitination of TAK1 (Y206 F) (Figure 6(k)), indicating that SHP-1 inhibits K63-linked ubiquitination of TAK1 by dephosphorylating Tyr 206 residue.

**β-arrestin 2 interferes TRAF6 association with TAK1**

TRAF6 recruits TAB2 by its K63-linked ubiquitin chain, which in turn binds to the TAK1/TAB1 complex and induces TAK1 phosphorylation and activation.14 As SHP-1 inhibits K63-linked polyubiquitination of TRAF6, we speculated that SHP-1 might inhibit the binding of TRAF6 and TAB2, thereby inhibiting the binding of TRAF6 and TAK1. Consistent with our expectation, SHP-1 inhibits TRAF6/TAB2 (Figure 6(a)) and TRAF6/TAK1 interactions (Figure 6(b)). Furthermore, we observed that β-arrestin 2 inhibited the association of endogenous TRAF6 with TAK1 and TAB2 in wild-type peritoneal macrophages infected by EPEC, whereas this inhibition disappeared when β-arrestin 2 or Tir was deleted (Figure 6(c)), indicating that β-arrestin 2 mediated the inhibitory effect of Tir on TLR signaling.

**Discussion**

EPEC Tir plays a crucial role in redirecting the cell’s structural components to support the attachment of EPEC.30 However, the role of Tir in immune response and the molecular mechanism underlying this process remain elusive. Here, we report that Tir inhibits the host cellular immune response via β-arrestin 2. β-arrestin 2 deficiency significantly diminishes the inhibitory effect of Tir. We demonstrated that β-arrestin 2 promotes the recruitment of SHP-1 to Tir and inhibits the activation of TRAF6 and TAK1, which is an essential negative regulator of the innate immune response.

The Toll-like receptors (TLRs) signaling pathway is essential in innate immunity. Upon the stimulation of pathogen-associated molecular patterns (PAMP) such as lipopolysaccharides (LPS),

[Figure 6. β-arrestin 2 interferes with the association of TRAF6 with TAK1.](#) (a,b) Immunoassay of lysates of HEK293 T cells expressing vectors encoding various proteins (above lanes). (c) IP and IB analysis of lysates of mouse primary macrophages cells of wild-type mice or β-arrestin 2 deficient mice, left uninfected (Medium) or infected with EPEC or EPEC Δtir for 4 h. WT, Arrb2+/+, KO, Arrb2−/−. Data are representative of at least three independent experiments.
TLR4 recruit myeloid differentiation primary response gene 88 (MyD88) and IRAK1/4, which then forms a complex with TRAF6 and triggers its K63-linked auto-ubiquitination. The K63-linked ubiquitin chains of TRAF6 recruit TAB2 form a complex with TAK1/TAB1 and activate TAK1, which then activates IkB kinase (IKK) complex, as well as the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), promoting the translocation of NF-κB and c-Fos/c-Jun to the nucleus and initiate proinflammatory cytokines production. On the basis of our *in vitro* and *in vivo* data, we propose the model as shown in Figure S8. β-arrestin 2 interacts with ITIM-phosphorylated Tir and enhances the recruitment of SHP-1, which inhibits the K63-linked ubiquitination and activation of TRAF6. In addition, reduced K63-linked ubiquitination of TRAF6 limits TAB2 binding, which inhibits TRAF6-TAK1 interaction and activation of TAK1. Meanwhile, SHP-1 interacts with TAK1, inhibits TAK1/TAB1 interaction, and reduces the phosphorylation and ubiquitination of TAK1. Overall, Tir inhibits host innate immunity through β-arrestin 2.

Beyond its traditional roles in mediating GPCR desensitization and endocytosis, β-arrestin 2 regulates multiple signal pathways. In general, it appears to encompass a fairly discrete set of functions by binding with diverse catalytically active nonreceptor proteins, such as cAMP phosphodiesterase, phosphoinositides, Src family nonreceptor tyrosine kinases, E3 ubiquitin ligases, deubiquitinases, and tyrosine phosphatase. β-arrestin 2 works as a scaffold to bring these enzymes to their targets, resulting in either promotion or inhibition of the signaling transduction. McDonald et al. reported that β-arrestin 2 acted as a scaffold protein, which changed the spatial distribution and activity of JNK3 and its kinase ASK1, inducing enhanced JNK3 phosphorylation. In our study, β-arrestin 2 functions as a scaffold protein to facilitate the recruitment of the negative regulator SHP-1 to TRAF6 and TAK1, which eventually leads to the inhibition of proinflammatory cytokine production.

The immune system is exquisitely balanced. Posttranslational modifications (PTMs) such as ubiquitination and phosphorylation play important roles to ensure the delicate balance underlying immune signal transduction. Ubiquitination and phosphorylation are tightly linked and could regulate each other in many cases. For example, K63-linked polyubiquitination of Connexin 43 is induced by its phosphorylation. Steroidogenic factor 1 (SF-1) controls sexual development in the embryo, ubiquitination of SF-1 requires its phosphorylation at Ser203. E3 ubiquitin ligase binds with phosphorylated β-catenin to mediate its ubiquitination, while mutation of a single serine prevents the ubiquitination. These studies show that phosphorylation could regulate ubiquitination. In our study, we found that SHP-1 inhibits K63-linked ubiquitination of TRAF6 and TAK1 by dephosphorylating their certain tyrosine residues, which provide a proof that phosphorylation regulates ubiquitination during the infection.

Some other bacterial proteins, such as EHEC Tir and *Helicobacter pylori* (HP) CagA, also contain ITIM or ITIM-like motifs. The CagA virulence motif “EPIYA” (a five-amino-acid sequence (Glu-Pro-Ile-Tyr-Alu)) shares partial sequence similarity with ITIMs (Figure S9A). By a bioinformatics prediction method, we found several virus proteins, such as HIV virulence protein Vif and Epstein–Barr virus (EBV) LMP2A, also share high similarity with the ITIM sequence (Figure S9A). Using Co-IP method, we found EBV LMP2A and EHEC Tir associates with β-arrestin 2. Moreover, the association happened only when pervanadate existed (Figure S9B, C), suggesting the interactions were dependent on the tyrosine phosphorylation of ITIM. It has been reported that after CagA was injected into host cells, the tyrosine in its “EPIYA” motif was phosphorylated, which is an essential process for the pathogenesis of HP. After infection with HP, we observed that endogenous β-arrestin 2 was coimmunoprecipitated with CagA (Figure S9D). Thus, the use of cellular ITIM or ITIM-like motifs by microbial proteins to interact with β-arrestin 2 and subvert the host immune signaling pathway could be a common strategy by which microbial pathogens establish a successful infection. Our results provide a new target for clinical therapy of such infectious diseases.

Activation of TLR signaling is precisely controlled, because prolonged or excessive activation...
could cause host tissue damages by excessive release of inflammatory factors. The immune system employs inhibitory receptors bearing one or more ITIMs to negatively regulate immune responses. Ligand engagement by inhibitory receptors results in the phosphorylation of ITIM by Src family tyrosine kinases and the recruitment of tyrosine phosphatases, such as SHP-1 and SHP-2, which are critical negative regulators in immune signaling pathway. The data obtained in our story showed that ITIM containing immune inhibitory receptors-β-arrestin 2-SHP-1/2 axis could be an important negative immune response regulator. The role of Tir in pedestal formation in human intestinal epithelial cells (IECs) was well studied. The binding of EPEC to IECs triggers the formation of disease-associated actin-rich structure called pedestal; this process is important for its pathogenicity. During the first step of infection, EPEC adheres to the host epithelium non-intimately. Upon delivery into the cell cytoplasm, Tir is inserted into the plasma membrane in a hairpin-loop conformation and exposes an extracellular loop that interacts with the bacterial surface protein Intimin. This binding facilitates extremely tight attachment and results in the clustering of Tir in the plasma membrane that contributes to the downstream signaling transduction, leading to the formation of the actin-rich pedestal. The frequency of pedestal formation induced by Y483 F/Y511 F double mutant was normal, while the pedestals were longer than the wild-type Tir. It has been reported that tyrosine phosphorylation of Y483 and Y511 amino acid residues within tandem ITIM-like sequences are essential for F-actin-pedestal formation by recruiting a host inositol phosphatase, SHIP2. Our results showed that β-arrestin 2 only bound with tyrosine-phosphorylated Tir at 483/511 amino acids. β-arrestin 2 is also involved in cytoskeletal rearrangement by interacting with kinases and phosphatases. Thus, we speculate that Tir-β-arrestin 2 complex may play roles in Tir inducing the formation of pedestals and A/E lesions in IECs after EPEC infection. Besides, Toll-like receptors (TLRs) are expressed diverse on IECs and are critical for intestinal homeostasis. Whether Tir-β-arrestin 2-SHP-1 axis functions in regulating the ability of IECs to release cytokines upon EPEC infection need to be further investigated. Taken together, our findings reveal that β-arrestin 2 is essential for Tir-mediated immune evasion, which might be a new target for the development of therapeutics to treat microbial infection.

Materials and methods

Mice

C57BL/6 and β-arrestin 2 deficient mice were bred in specific pathogen-free conditions at Shanghai Research Center for Model Organisms. All animal experiments were undertaken in accordance with the Institutional Animal Care and Use Committee of Fudan University.

Cell culture

HEK293 T cells, RAW264.7 cells, and mouse primary macrophages were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone) supplemented with 10% (v/v) FBS (Gibco) and 100 U/ml penicillin and streptomycin. The HeLa cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS (Gibco) and 100 U/ml penicillin and streptomycin.

Bacterial strains

EPEC, EPEC Δtir and EPEC (Δtir + HA-tir) were cultured for 12 h at 37°C in Luria-Bertani medium, supplemented with 100 μg/ml of ampicillin. EPEC (Δtir + HA-tir) was supplemented with 25 μg/ml kanamycin in addition. Before infection, EPEC and its mutants were cultured in DMEM supplemented with 10 mM HEPES (pH 7.4) in 5% CO2 for 12 h. C. rodentium strain (ATCC 51459; American Type Culture Collection) and its Tir deletion mutant were cultured in LB without antibiotics. TBK1 and BL21 strains were cultured in LB and induced by Isopropyl β-D-thiogalactoside (IPTG).

Isolation of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated as described. Peritoneal cavity cells were harvested
by peritoneal lavage using ice-cold Ca\(^{2+}\) and Mg\(^{2+}\)-free PBS, and then cultured in RPMI medium containing 10% (v/v) FBS (Gemini Bio Products), 1% ampicillin and streptomycin (Life Technologies) at 37°C in 95% air and 5% CO\(_2\) for 4 h. After the removal of non-adherent cells by washing, the adherent cells were macrophages. For in vitro infection assays, the macrophages were cultured in serum-free medium for 12 h to obtain quiescent cells and then infected with EPEC or its mutants for a different time. 2 × 10\(^6\) peritoneal macrophages were used for each infection. Multiplicity of infection (MOI) = 10. RAW264.7 cells and Caco-2 cells were infected in the same way.

**Oral infection, CFU counts, and histology**

C. rodentium and C. rodentium Δtir mutant were prepared and orally gavaged into C57Bl/6 J or β-arrestin 2\(^{-/-}\) mice. Mice were fasted for 4 h before the oral inoculation with 2 × 10\(^8\) CFU wild-type C. rodentium and its Δtir mutant in a total volume of 100 µl per mouse. After gavaging, the mice were fasted for 2 h. Body weights were monitored daily after infection. According to the experimental arrangement, fecal specimens were collected, weighed, homogenized in PBS, and plated on MacConkey agar plates for CFU analysis. Bacterial colonies were counted after overnight incubation at 37°C. Colonos and spleens were removed aseptically, weighed, and homogenized in PBS, and the CFUs were analyzed. Some colon and spleen tissues were retained for RT-PCR to detect cytokine production. The colons were dissected from the mice and fixed in 4% (v/v) paraformaldehyde. Paraffin-embedded tissue sections were stained with hematoxylin and eosin for evaluation of the tissue pathology.

**Plasmids and plasmid construction**

β-arrestin 2 cDNA was obtained from Dr. G. Pei (Tongji University, Shanghai, China). Flag-tagged or GST-tagged Tir (WT, Y483 F, Y511 F, Y483 F/ Y511 F) were constructed as described previously. Expression vectors for TAK1, TAB1, and TAB2 were kindly gifted by Dr. B. Ge (Tongji University, Shanghai, China).

**Transfection and immunoprecipitation**

HEK293 T cells were transiently transfected using polyethyleneimine (PEI). 48 h later, cells were treated for 20 min at 37°C with pervanadate (0.1 mM sodium orthovanadate and 10 mM H\(_2\)O\(_2\)) and washed with PBS, and then lysed in lysis buffer (Beyotime) supplemented with 1% Protease Inhibitor Cocktail (B14002; Selleck), 1 mM NaF and 1 mM Na\(_3\)VO\(_4\). Cell lysates were incubated overnight at 4°C with anti-Flag M2 Affinity Gel Beads. The beads were washed with PBST (1× PBS, 1% TritonX-100) three times and boiled for 10 min in SDS loading buffer.

**Confocal microscopy**

HeLa cells were transfected with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). 48 h later, the cells were infected for 4 h with JPN15 Δtir or JPN15 (Δtir + HA-tir), and then fixed for 30 min at 25°C with 4% formaldehyde. The cells were stained and examined with a Leica confocal microscope equipped with analytical software.

**GST pull down**

The GST-tagged Tir plasmids and its mutants Y483 F, Y511 F, Y483 F/Y511 F were transformed into TBK1 strains and induced to express either phosphorylated wild-type Tir or its point mutants. Recombinant proteins were purified as glutathione S-transferase (GST) fusion proteins to determine whether ITIM phosphorylation was required for direct interaction between Tir and β-arrestin 2.

**RNA interference**

β-arrestin 2 specific siRNA (5’-GGAACUCUG UGCGCUUAUTT-3’) were used to knock down the expression of endogenous β-arrestin 2, which was transfected into RAW264.7 cells by the Amaxa program D-032 and the Cell Line Nucleofector Kit V with the Amaxa Nucleofector apparatus according to the manufacturer’s instructions (Amaxa Biosystems). The ‘nonsense’ sequence (5’-UUCUCCGAACGUGACACGUTT-3’) was used as a control siRNA.
RT-PCR analysis

Total RNA was extracted with 1 ml of Trizol reagent according to the manufacturer’s instructions (Invitrogen). One μg total RNA was reverse-transcribed with the Rever Tra Ace qPCR RT Kit (FSQ-101; ToYoBo) according to the manufacturer’s instructions. The SYBR GREEN RT-PCR Kit (QPK-212; ToYoBo) was used for quantitative real-time RT-PCR analysis. Expression values were normalized to those obtained with the control gene gapdh (encoding glyceraldehyde phosphate dehydrogenase). All primers used are listed in Table S1.

Luciferase assay

HEK293 cells were co-transfected with pNF-κB-luc, an NF-κB reporter plasmid, or pAP-1-luc, an AP-1 reporter plasmid, pRL-TK-luc, and other plasmids. In TRAF6 related luciferase assays, after transfection for 48 h, the cells were induced by TNF-α (10 ng/ml) for 6 h or phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) for 12 h, and lysed for 15 min on ice. In TAK1 related luciferase assays, cells were lysed after cotransfection for 48 h. The lysate was analyzed using the Dual-Luciferase Reporter Assay System (Beyotime Biotechnology). Results are presented as the average of three measurements.

ELISA

TNF, IL-6, IL-12, IL-1β (cleaved active form) in the medium culturing peritoneal macrophages, which were infected by EPEC or its mutant for a certain time, were assayed according to manufacturer’s instructions (Dakewe Biotech Co., Ltd).

Statistical analysis

As indicated in the Figure legends, each experiment was performed independently for at least three times. Statistical analyzes were conducted using GraphPad Prism (Version 8.3.0). Error bars indicated standard deviations. Densitometry quantification was made with ImageJ Software. P values were calculated using two-way ANOVA. P values smaller than 0.05 were considered statistically significant.

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Author contributions

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Disclosure of Potential Conflicts of Interest

The authors declare that no competing interests exist.

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Ethics

Animal experimentation: The animal experiments were performed in accordance with National Institutes of Health guidelines for housing and care of laboratory animals and according to institutional regulations after protocol review and approval by the Institutional Animal Care and Use Committee of Fudan University (Protocol Number 20160225-049).
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