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Epidermal Growth Factor Receptor–Responsive Indoleamine 2,3-Dioxygenase Confers Immune Homeostasis During *Shigella flexneri* Infection

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The resolution of *Shigella flexneri* infection–associated hyperinflammation is crucial for host survival. Using in vitro and in vivo models of shigellosis, we found that *S. flexneri* induces the expression of indoleamine 2,3-dioxygenase 1 (IDO1) through the nucleotide oligomerization domain 2 (NOD2) and epidermal growth factor receptor (EGFR) signaling pathway. Congruently, abrogation of NOD2 or EGFR compromises the ability of *S. flexneri* to induce IDO1 expression. We observed that the loss of IDO1 function in vivo exacerbates shigellosis by skewing the inflammatory cytokine response, disrupting colon epithelial barrier integrity and consequently limiting the host life-span. Interestingly, administration of recombinant EGF rescued mice from IDO1 inhibition–driven aggravated shigellosis by restoring the cytokine balance and subsequently restricting bacterial growth. This is the first study that underscores the direct implication of the NOD2-EGFR axis in IDO1 production and its crucial homeostatic contributions during shigellosis. Together, these findings reveal EGF as a potential therapeutic intervention for infectious diseases.

Keywords. *Shigella flexneri*; inflammation; EGFR signaling; IDO1; immune homeostasis; enteropathy.

Infection with pathogenic members of the genus *Shigella* remains an unsolved global concern, forming the second leading cause of diarrheal deaths across all ages in 2016 [1]. These organisms are highly infectious, with a miniscule number of 10 bacteria being sufficient to manifest disease. *Shigella* invades the host through intestinal epithelial cells, eventually reaching the underlying macrophages and mucosal membrane [2] and facilitating further dissemination. In response, the host mounts a gamut of strongly proinflammatory protective immune responses, such as interleukin 1β and interleukin 8 secretion, which lead to the expression of antimicrobial peptides and drive the recruitment of polymorphonuclear cells, further disrupting epithelial barrier integrity and enhancing pathogen invasion [3]. In addition to the inflammatory response, interaction with *Shigella* triggers major alterations in the metabolic and physiological status of the host cells. Several energy-sensing pathways, including the mTOR pathway, are deregulated upon the onset of infection, which directly correlates with cellular autophagic processes [4]. Interestingly, recent reports have highlighted the ability of the pathogen to circumvent these major immune assaults, including phagosome entrapment, proinflammatory cytokine burst, and expression of antimicrobial peptides by intercepting host cell signaling pathways, thereby modulating host membrane, cytosol, and chromatin components [5, 6]. Despite these survival advantages, infection with *Shigella* progresses to a debilitating state primarily when the host is immunocompromised [7], suggesting crucial roles for host factors in controlling shigellosis.

The alarming rise of multidrug-resistant strains of *Shigella* and the lack of appropriate medical interventions and vaccines reveals the necessity to understand shigellosis at cellular and molecular levels, to identify suitable targets for prophylactic and therapeutic strategies [8]. By using a mouse model of *Shigella flexneri* infection, we assessed the contribution of the host signaling network in moderating the extent of shigellosis. Interestingly, *Shigella*-driven inflammation is countered by the host to maintain cellular homeostasis and protect against autoimmune damage. Host effectors such as PGRP, RUNX3, and NRF2 are known antiinflammatory mediators [9–11]. Intriguingly, it could be noticed from the literature that inhibition of one such mediator, indoleamine 2,3-dioxygenase (IDO), during intestinal bowel disease leads to severe disease pathology [12]. Further, this tryptophan-metabolizing enzyme was also reported to be highly expressed in Crohn disease [13]. IDO1 has been documented to maintain gut homeostasis by limiting inflammatory responses through the modulation of T-cell functions [14]. However, its implications in infections have not been extensively studied, and the molecular mechanisms governing its production and the downstream effectors that lead to its homeostatic attributes during infections remain elusive. Through this study, we delineate the yet unexplored role for IDO1 in maintaining immune homeostasis during *Shigella* infection.

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We show that *Shigella* infection–driven IDO1 expression is finely regulated by the nucleotide oligomerization domain 2 (NOD2)–epidermal growth factor receptor (EGFR)–c-Abl tyrosine kinase–WNT signaling axis at the molecular level. Findings of clinical trials and mouse models involving EGFR inhibition correlate with poor prognosis for intestinal inflammation. Further, the role for the cytosolic receptor NOD2 in shigellosis is largely underappreciated. We found that IDO1-dependent homeostatic function is furnished by its modulatory effect on inflammatory mediators: inhibition of IDO1 led to a compromised expression of infection-induced antiinflammatory mediators while exaggerating the effects of the proinflammatory arm. Corroborating our observations, we found that mice that received the IDO1 inhibitor 1-methyl-D-tryptophan (1MT) and infected with *S. flexneri* died from the immune insult much earlier than those that did not receive 1MT. Interestingly, supplementation with recombinant EGF could augment the resistance of mice to *S. flexneri* infection–driven disease pathology, irrespective of the presence of IDO1 inhibitor. Taken together, we propose that, despite the highly evolved strategy for infection, EGFR signaling–driven IDO1 production in the context of *S. flexneri*–associated intestinal inflammation is cardinal to sustaining immune equipoise.

MATERIALS AND METHODS

Mice and Cells

BALB/c mice were purchased from The Jackson Laboratory and maintained at the Central Animal Facility, Indian Institute of Science (Bangalore, India). The RAW 264.7 mouse macrophage–like cell line was obtained from the National Centre for Cell Sciences (Pune, India). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (Supplementary Materials).

Ethics Statement

All studies involving mice were in accordance with the Institutional Animal Ethics Committee for animal experimentation. Protocols for the care and use of animals accorded with the national guidelines of the Committee for the Purpose of Control and Supervision of Experiments and Animals, Government of India (Supplementary Materials).

Bacteria

S. flexneri cultured in Luria-Bertani broth was grown to mid log phase and used at a multiplicity of infection (MOI) of 10 for in vitro experiments. *Enterobacter faecalis* was cultured in brain heart infusion medium and used for infection (Supplementary Materials).

Treatment With Pharmacological Reagents and Transient Transfection Studies

Cells were pretreated with the requisite pharmacological inhibitors (as indicated in the respective experiments) for 1 hour, followed by infection with *S. flexneri*/*E. faecalis*, treatment with muramyl dipeptide (MDP); or treatment with recombinant EGF, and processed for transcript and protein level analyses. Transfection in peritoneal macrophages or RAW 264.7 cells was performed using the polyethyleneimine-based method, and transfected cells were subjected to experiment-specific indicated treatments (Supplementary Materials).

RNA Isolation and Real-Time Quantitative PCR (qPCR)

Total RNA from treated cells was isolated using the guanidinium thiocyanate–phenol–chloroform extraction method. An equal amount of RNA was converted into complementary DNA (cDNA), using oligodT primers and MoMuLV reverse transcriptase. The cDNA thus obtained was used for SyBR green–based real-time qPCR analysis (Supplementary Materials).

Immunoblotting

Treated cells were lysed, and an equal amount of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer onto polyvinylidene fluoride membrane and probing for the requisite molecules, using specific primary and secondary antibodies (Supplementary Materials).

Chromatin Immunoprecipitation Assay

Treated cells were fixed, and chromatin was sheared by sonication. After immunoprecipitation with specific antibodies, chromatin was eluted, purified, and subjected to real-time qPCR for specific promoters (Supplementary Materials).

Experimental *S. flexneri*/*E. faecalis* Infection Model

In vivo experiments with *S. flexneri*/*E. faecalis* were performed in accordance with previously published protocols [15, 16]. Briefly, mice were administered with 1MT intraperitoneally for 12 hours [17], followed by infection with 5×10^8 colony-forming units (CFUs) of *S. flexneri* (via the intraperitoneal route)/*E. faecalis* (via the intravenous route). After 1 hour and 12 hours of infection, 3 µg of EGF was injected intraperitoneally per mouse [18]. After 18 hours of infection (for *S. flexneri*), mice were euthanized; colon tissue specimens were isolated and processed for RNA collection, in vivo CFU analyses and evaluation by immunoblotting, enzyme-linked immunosorbent assay, hematoxylin and eosin staining. For mice infected with *E. faecalis*, the colon was assessed for length and bacterial CFUs after 48 hours of infection.

The in vivo intraperitoneal shigellosis model and intravenous *E. faecalis* model do not completely mimic the natural oral-fecal route of infection and invasion across the intestinal epithelia, and they also bypass the mucosal immune response. However, a comparison of different shigellosis models in mice has demonstrated significant similarity to human shigellosis when infected intraperitoneally, as it allows assessment at the primary site of infection (ie, the intestine). Also, intravenous *E. faecalis* infection model has been used previously for testing the virulence of different strains of the pathogen, and many accept that the

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intestinal dysfunction it causes is similar to that during human infection with the same pathogen (Supplementary Materials).

Statistical Analysis

Statistical significance was determined on the basis mean values ± standard errors from ≥3 independent experiments, and P values of < .05 were defined as significant. GraphPad Prism 5.0 software was used for all statistical analyses (Supplementary Materials).

RESULTS

S. flexneri Infection Induces EGFR-Dependent IDO1 Expression

As described above, infection with *S. flexneri* culminates in intestinal dysregulation stemming from hyperinflammation. The EGFR pathway has been found to be instrumental in maintaining the cytokine balance during experimental colitis [19], and its inhibition is strongly correlated with gastrointestinal toxicity [20]. Also, EGFR has recently been shown to be activated by *S. flexneri* infection [21]. However, its definitive role in the establishment of *S. flexneri*–triggered inflammatory responses remains elusive. We confirmed that *S. flexneri* infection led to robust induction and activation of the EGFR pathway (Supplementary Figure 1A and Figure 1A) and concomitantly observed enhanced NOD2 signaling activation (Supplementary Figure 1B) and augmented expression of inflammatory mediators (Supplementary Figure 1C). We further analyzed the possible involvement of NOD2 in *S. flexneri*–induced EGFR activation and found that in vivo administration of MDP, a NOD2 agonist, elicited marked induction and activation of the EGFR pathway (Supplementary Figure 1D and 1E). We speculated whether *S. flexneri*–orchestrated EGFR activation is essential for the expression of the crucial regulator of intestinal inflammation, IDO1.

We found enhanced expression of *Ido1* in macrophages infected with *S. flexneri* (Figure 1B). Although *S. flexneri* is known to induce macrophage death early during infection, careful time kinetics experiments demonstrated that the observed IDO1 expression did not result from macrophage death, as *S. flexneri* failed to induce host pyroptotic cell death in the absence of lipopolysaccharide priming (Supplementary Figure 1F). Subsequently, it was found that in vitro loss of EGFR signaling by specific pharmacological inhibitors (Gefitinib and AG1478) compromised the ability of *S. flexneri* to induce the expression of IDO1 (Figure 1C). Also, in vivo administration of recombinant EGF in mice led to enhanced IDO1 expression (Figure 1C). Colon length offers a direct measure of inflammation during *S. flexneri* infection [15, 22]. However, we observed no significant differences in the colon length of mice treated

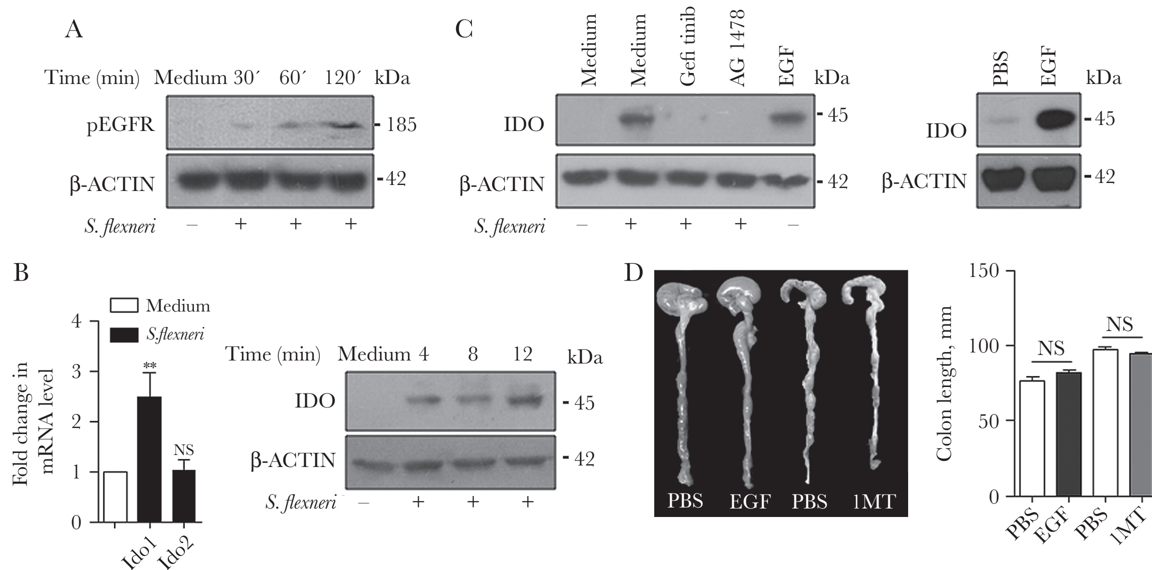

Figure 1. *Shigella flexneri* infection leads to epidermal growth factor receptor (EGFR)–dependent indoleamine 2,3-dioxygenase 1 (IDO1) expression. *A*, Peritoneal macrophages (PMs) were infected with *S. flexneri* (multiplicity of infection, 10) for the indicated times to assess the activation of EGFR signaling by immunoblotting. *B*, PMs were infected with *S. flexneri* for 12 hours to assess the expression of IDO at the transcript level by real-time quantitative polymerase chain reaction (qPCR; *left*) or for the indicated times to analyze the expression of IDO at the protein level by immunoblotting (*right*). *C*, PMs were treated for 12 hours with recombinant murine EGF (50 ng/mL) or pretreated with gefitinib (an EGFR inhibitor; 20 µM) or AG1478 (an EGFR inhibitor; 5 µM) as indicated, followed by 12 hours of *S. flexneri* infection, and cells were harvested and analyzed for the expression of IDO at the protein level by immunoblotting (*left*). Mice were treated with recombinant murine EGF (3 µg/mouse) for 18 hours, and IDO expression was assessed in colon homogenates by immunoblotting (*right*). *D*, Mice were treated with recombinant murine EGF (3 µg/mouse) or 1-methyl-D-tryptophan (1MT; an inhibitor of IDO1; 10 mg/mouse) for 18 hours, and colon length was estimated from the respective groups of mice. Two controls treated with phosphate-buffered saline (PBS) have been used for 2 distinct groups of mice. Five mice were used per group. The experiment was performed in biological triplicates. All immunoblots are representative of 3 independent experiments. β-actin was used as the loading control. Real-time qPCR data represent means ± standard errors (n = 3). See also Supplementary Figure 1. NS, not significant. $^{**}P < .005$, by the Student t test.

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with recombinant EGF or 1MT (Figure 1D). These observations suggest possible infection-specific contributions of EGFR-IDO1 during *S. flexneri* pathogenesis.

Inhibition of IDO1 Leads to Exacerbated Shigellosis

To assess the relevance of *S. flexneri*–induced EGFR-IDO1 in vivo, we used an intraperitoneal model for shigellosis (Figure 2). In consonance with this previously described model [15], histopathological analysis of colon sections obtained from *S. flexneri*-infected mice showed a partial loss of epithelial barrier integrity, a blunting of crypts (Figure 2A), and an associated reduction in colon length (Figure 2B) and host survival (Figure 2C). With indications that IDO serves key antiinflammatory [23] and antimicrobial [24] functions, exacerbated disease pathology was observed in infected mice administered with 1MT [17], with a further deterioration in host median life-span (Figure 2C). We observed a significant reduction in colon length (Figure 2B) and excessive accumulation of goblet cells in infected mice pretreated with 1MT. Goblet cells, which secrete mucins, are essential to develop an anatomical barrier against invading pathogens [25]. But despite the increased number of goblet cells, we also observed a concomitant increase in the bacterial burden upon 1MT treatment (Figure 2D). This can be explained by compromised mucin production or development of a defective inner mucus layer during hyperinflammation, which would permit bacterial invasion and pathogenesis [26]. Taken together, IDO1 was found to be crucial for maintaining immune homeostasis during *S. flexneri* infection.

Next, the premise that the EGFR signaling axis plays a role in the production of IDO1 led us to explore a potential rescue from disease pathology upon administration of EGF in mice

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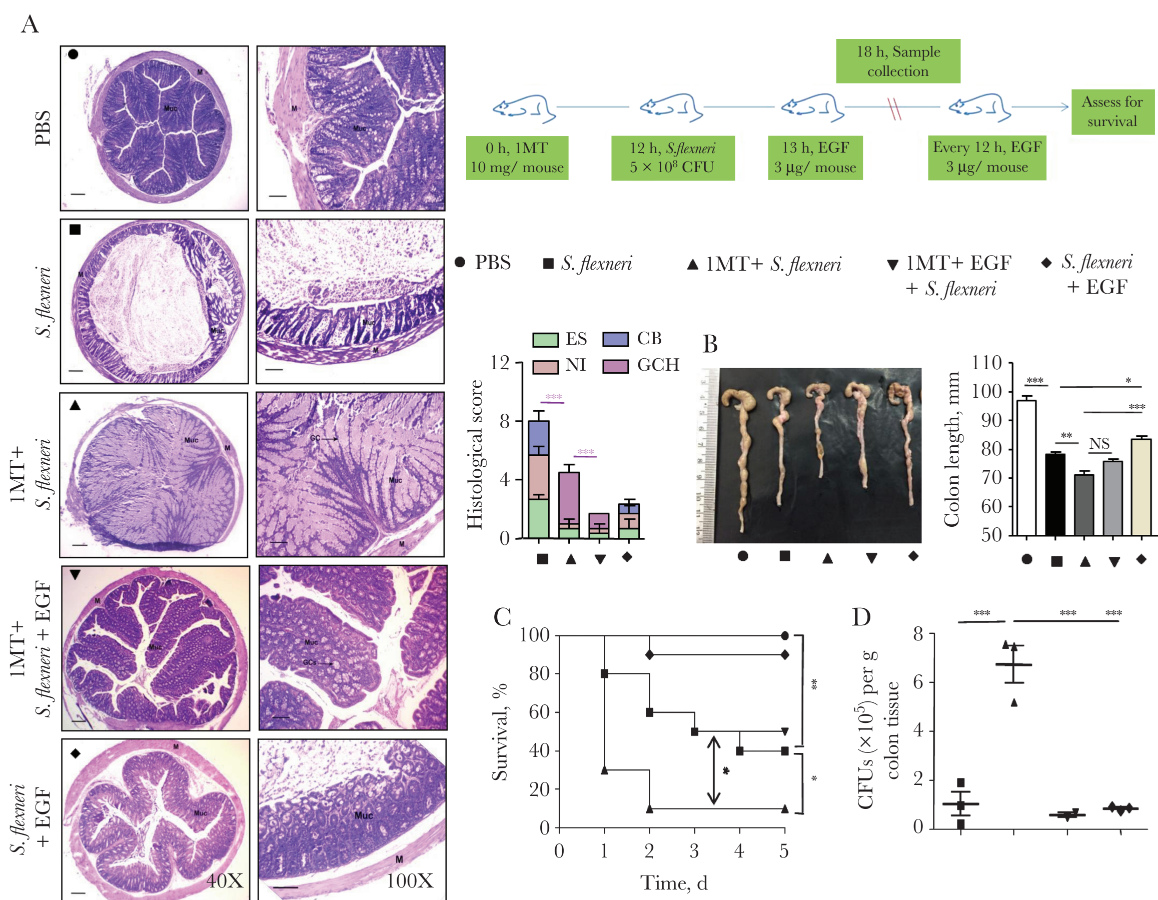

Figure 2. Epidermal growth factor (EGF) ameliorates exacerbated shigellosis during indoleamine 2,3-dioxygenase (IDO) inhibition. To create an in vivo shigellosis model, Balb/c mice were administered 1-methyl-D-tryptophan (1MT; an inhibitor of IDO1; 10 mg/mouse) intraperitoneally 12 hours before intraperitoneal infection with *Shigella flexneri* (5×10^8), followed by administration of recombinant murine EGF (3 µg/mouse) at 1 hour and every 12 hours after infection. *A*, *B*, and *D*, Treated mice were euthanized 18 hours after infection or at death and underwent histopathological analysis with hematoxylin-eosin stain (*A*), analysis of colon length (*B*), and determination of *S. flexneri* colony-forming units (CFUs; *D*). *C*, Kaplan-Meyer curve depicting the percentage survival of mice, by treatment condition. A minimum of 5 mice were used per experimental group for histopathological analysis, and 10 mice were monitored for survival. Histological findings were scored using 4 parameters: epithelial shedding (ES; score, 0–4), neutrophil infiltration (NI; score, 0–4), blunting of crypts (CB; score, 0–4), and goblet cell hyperplasia (GCH; score, 0–4). Control mice did not show any pathology (score, 0). Scale bar, 200 µm for 40× original magnification and 100 µm for 100× original magnification. NS, nonsignificant; PBS, phosphate-buffered saline. $^{*}P < .05$, $^{**}P < .005$, and $^{***}P < .001$ by 1-way analysis of variance followed by the Bonferroni post hoc test.

treated with 1MT. Intriguingly, EGF imparted no significant rescue in the colon length of 1MT-treated infected mice at the time point tested (Figure 2B). However, there was a distinct survival benefit conferred to mice treated with EGF in these conditions (Figure 2C). An associated reduction in the bacterial burden as compared to that for IDO1 inhibitor-treated mice was evident upon therapeutic administration of EGF (Figure 2D). The histopathological scores were found to corroborate with survival statistics. Exacerbated shigellosis-associated disrupted epithelial barrier integrity and goblet cell hyperplasia was minimally observed in mice receiving EGF therapy (Figure 2A). Interestingly, we found that IDO1 is also crucial for conferring immune equipoise during infection with another enteric pathogen, *E. faecalis*. Akin to *S. flexneri*, *E. faecalis* infection led to EGFR-dependent IDO1 expression (Supplementary Figure 2A). Perturbation of IDO1 function in *E. faecalis*–infected mice compromised mice survival (Supplementary Figure 2B), led to reduction in colon length (Supplementary Figure 2C), and resulted in an elevated *E. faecalis* burden in the colon (Supplementary Figure 2D). Further, these manifestations were alleviated by treatment with recombinant EGF (Supplementary Figure 2B-D). These results clearly suggest the homeostatic ability of IDO1 during infection with distinct enteric pathogens, which is lost upon its inhibition, and demonstrate a therapeutic value for EGF under such conditions.

NOD2 Drives EGFR-Dependent IDO1 Expression During *S. flexneri* Infection

NOD2 signaling is an intracellular immune surveillance pathway, crucial for regulating inflammation. However, its implication in *S. flexneri*–associated inflammatory responses is not appreciated. As NOD2 ligand led to the activation of EGFR signaling (Supplementary Figure 1D, E), we speculated a possible role for *S. flexneri*–induced NOD2 in driving IDO1 expression. We found induced expression of IDO1 in the splenocytes of mice treated with MDP (Supplementary Figure 1G, H). Further, the involvement of NOD2 was specifically addressed with the failure of *S. flexneri* to induce IDO1 production in NOD2–knocked down macrophages (Figure 3*A*). The canonical NOD2 signaling pathway results primarily in nuclear factor κB (NF-κB)–driven gene expression [27], which led us to analyze the dependence of IDO1 expression on the transcription factor NF-κB. It was found that pharmacological inhibition of NF-κB compromised the ability of *S. flexneri* to induce *Ido1* expression (Figure 3B). However, further validation of the recruitment of NF-κB over the promoter of *Ido1* at a specific NF-κB–binding site (site 1) or at random sites spanning the 2-kb promoter region (sites 2, 3, and 4) during MDP treatment or *S. flexneri* infection did not correlate with results of the transcript analysis (Figure 3C). These observations pointed toward an indirect regulation of IDO1 expression upon NOD2 stimulation. At this conjecture, the possible nexus of NOD2-driven IDO1 expression with EGFR was assessed. We found that the induction of the *Egf* transcript was dependent on NF-κB as inhibition of NF-κB compromised *S. flexneri*-induced *Egf* expression (Figure 3D). Further, we found augmented recruitment of NF-κB on the promoter of *Egf* upon *S. flexneri* infection or MDP treatment (Figure 3C). These observations indicated that NOD2 uses EGFR signaling to induce IDO1 expression during *S. flexneri* infection. This was further corroborated by the compromised expression of MDP-induced IDO1 in the presence of EGFR inhibitors (Gefitinib/AG1478) (Supplementary Figure 1I).

β-Catenin Effectuates EGFR-Mediated Expression of IDO1

EGFR interacts with various adaptors and signaling intermediates upon stimulation [28]. One of the well-established networks involves the WNT pathway [29], which had been strongly implicated in inflammatory dysregulation driven by NOD2 [30–33]. WNT signaling was found to be responsive to EGFR during *S. flexneri* infection or MDP stimulation because blockage of EGFR signaling compromised activation of the WNT pathway (Figure 4A and Supplementary Figure 3*A*). We found WNT signaling to be a regulator of *S. flexneri*–induced IDO1 expression, because perturbing WNT pathway with specific inhibitors (FH535 and IWP-II; Figure 4B and Supplementary Figure 3*B*) or overexpressing a TCF4 dominant-negative construct (Figure 4B) abrogated IDO1 expression. Small interfering RNA–mediated β-catenin knockdown was also found to compromise the ability of *S. flexneri* to induce IDO1 expression (Figure 4C). Corroborating this observation, macrophages overexpressing β-catenin showed significantly elevated levels of IDO1 (Figure 4B). Further validation with a chromatin immunoprecipitation assay confirmed the occupancy of the *Ido1* promoter by β-catenin upon *S. flexneri* infection or MDP treatment (Figure 4D). This occupancy also occurred in the presence of EGF (Figure 4D), endorsing EGFR-mediated β-catenin–dependent expression of IDO1.

c-Abl Mediates EGFR-WNT–Driven IDO1 Expression Through Homeodomain Interacting Protein Kinase 2 (HIPK2) Serine-Threonine Kinase

The activation of the WNT pathway is a multipronged phenomenon, encompassing several alternative intermediates [34]. EGFR (a receptor tyrosine kinase) is incapable of directly inactivating GSK-3β by Ser phosphorylation. Interestingly, we found evidence in the literature supporting an association between HIPK2, a serine-threonine kinase, and components of the WNT pathway that results in modulation of the signaling outcome [35]. Of interest, inhibition of HIPK2 was associated with enhanced production of proinflammatory molecules, suggesting its role in immune functions [36]. HIPK2 activation requires tyrosine phosphorylation brought about by self-regulatory mechanisms or tyrosine kinase–dependent processes [37, 38]. c-Abl, a nonreceptor tyrosine kinase, is reported to complement the

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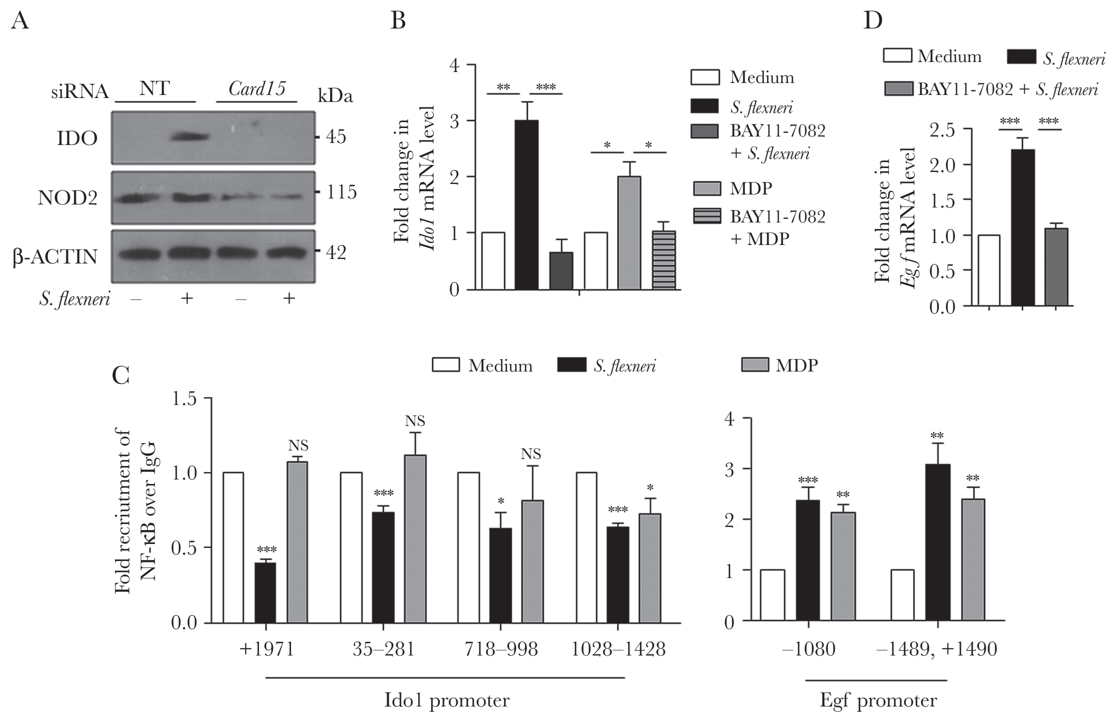

Figure 3. Nucleotide oligomerization domain 2 (NOD2) drives epidermal growth factor receptor (EGFR)–dependent indoleamine 2,3-dioxygenase 1 (IDO1) expression during *Shigella flexneri* infection. *A*, Peritoneal macrophages (PMs) were transfected with Card15 or nontargeting (NT) small interfering RNA (siRNA). Transfected cells were infected with *S. flexneri* for 12 hours, and whole-cell lysates were assessed for IDO expression and knock down of NOD2 by immunoblotting. *B* and *D*, PMs were pretreated with BAY11-7082 (an inhibitor of nuclear factor κB [NF-κB]; 10 μM) for 1 hour, followed by *S. flexneri* infection for 12 hours, to analyze the transcript level of *Ido1* (B) and *Egf* (D) by real-time quantitative polymerase chain reaction (qPCR). *C*, PMs were infected with *S. flexneri* or treated with muramyl dipeptide (MDP; a NOD2 agonist) for 8 hours, followed by analysis of chromatin for the recruitment of NF-κB over the *Ido1* promoter (*left*) and *Egf* promoter (*right*) by a chromatin immunoprecipitation assay. The immunoblot is representative of 3 independent experiments. β-actin was used as a loading control. Real-time qPCR data represent means ± standard errors (n = 3). See also Supplementary Figure 1. IgG, immunoglobulin G; NS, not significant. *$P < .05$, **$P < .005$, and ***$P < .001$ by the Student t test (*C*) and 1-way analysis of variance followed by the Bonferroni post hoc test (*B* and *D*). .

activities of EGFR [39], and, importantly, Abl kinases have been implicated in infection by *S. flexneri* [40]. We found that perturbation of c-Abl by using the pharmacological inhibitor imatinib or overexpression of a c-Abl kinase dead construct abrogated *S. flexneri*– or MDP-induced WNT signaling (Figure 5A and Supplementary Figure 3C) and subsequent IDO1 expression (Figure 5B and Supplementary Figure 3*D*). Further, c-Abl was found to use HIPK2 to bring about inhibitory phosphorylation on GSK-3β and activate WNT signaling. Corroborating these findings, loss of function of HIPK2 led to compromised WNT signaling activation (Figure 5C) and IDO1 expression in the presence of *S. flexneri* infection (Figure 5D) or MDP stimulation (Supplementary Figure 3*E* and 3*F*). Conversely, HIPK2 wild-type overexpression alone also induced IDO1 production (Figure 5D). These observations illustrate a finely regulated orchestration of serine/threonine kinases and tyrosine kinases to bring about the expression of IDO1 during *S. flexneri*–induced NOD2-driven inflammatory responses.

IDO1 Maintains Homeostasis by Modulating the Production of Inflammatory Mediators

To assess the possible mechanisms that could contribute to the previously observed ability of IDO1 to confer immune equipoise (Figure 2), a panel of inflammatory mediators was analyzed. It has been documented that the deficiency of antiinflammatory mediators such as interleukin 10 leads to an ineffective mucin barrier [26], as is also speculated in the current study. *S. flexneri* infection was found to induce the production of proinflammatory and antiinflammatory mediators. Further, inhibition of IDO1 by 1MT compromised infection-driven production of antiinflammatory molecules (interleukin 10, interleukin 4, transforming growth factor β, and Arg1), with concomitantly enhanced expression of proinflammatory cytokines (interleukin 12, interleukin 1β, interleukin 17, and interferon γ), both at the transcript level (Figure 6A) and the protein level (Figure 6B). These results indicate a novel role for IDO1 in calibrating the outcome of infection by differentially regulating the expression of inflammatory mediators.

Also, since IDO1 is found to be cardinal in the regulation of inflammatory mediators, we analyzed whether the observed salvage with EGF (Figure 2) could be correlated with the recovery of

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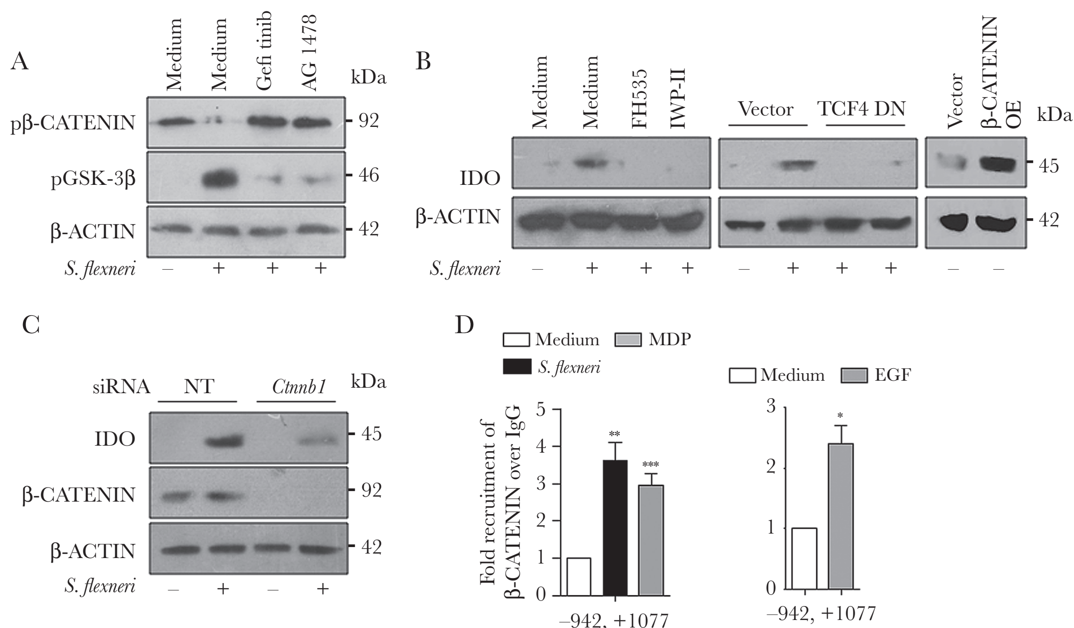

Figure 4. Epidermal growth factor receptor (EGFR) signaling is involved in cross talk with β-catenin to drive indoleamine 2,3-dioxygenase 1 (IDO1) expression. *A*, Murine peritoneal macrophages (PMs) were treated with the EGFR inhibitors gefitinib (20 μM) and AG1478 (5 μM) for 1 hour, followed by 1 hour of *Shigella flexneri* infection (multiplicity of infection, 1:10), and whole-cell lysates were analyzed for the activation of WNT signaling by immunoblotting. *B*, PMs were pretreated with the WNT inhibitors FH535 (15 μM) and IWP-II (5 μM) for 1 hour, followed by *S. flexneri* infection for 12 h, and the expression of IDO was analyzed at the protein level by immunoblotting (*left*). RAW 264.7 macrophages were transfected with TCF4 dominant-negative (DN) or β-catenin overexpression (OE) constructs for 8 hours. Transfected cells were infected with *S. flexneri* for 12 hours where indicated, and whole-cell lysates were assessed for IDO expression by immunoblotting (*middle* and *right*). *C*, Mouse PMs were transfected with *Ctnnb1* small interfering RNA (siRNA; 100 nM) or nontargeting (NT) siRNA (100 nM) for 8 hours, followed by recovery for 24 hours. Transfected cells were then infected with *S. flexneri* for 12 hours, and whole-cell lysates were assessed for IDO expression and knock down of β-catenin by immunoblotting. *D*, Murine PMs infected with *S. flexneri* (multiplicity of infection,10), stimulated with muramyl dipeptide (MDP), a nucleotide oligomerization domain 2 agonist (100 ng), or treated with murine recombinant EGF (50 ng/mL) were assessed for the occupancy of β-catenin over the *Ido1* promoter, by a chromatin immunoprecipitation assay. All immunoblots are representative of 3 independent experiments. β-actin was used as a loading control. Real-time quantitative polymerase chain reaction (qPCR) data represents means ± standard errors (n = 3). See also Supplementary Figure 2. *$P < .05$, **$P < .005$, and ***$P < .001$ by the Student *t* test.

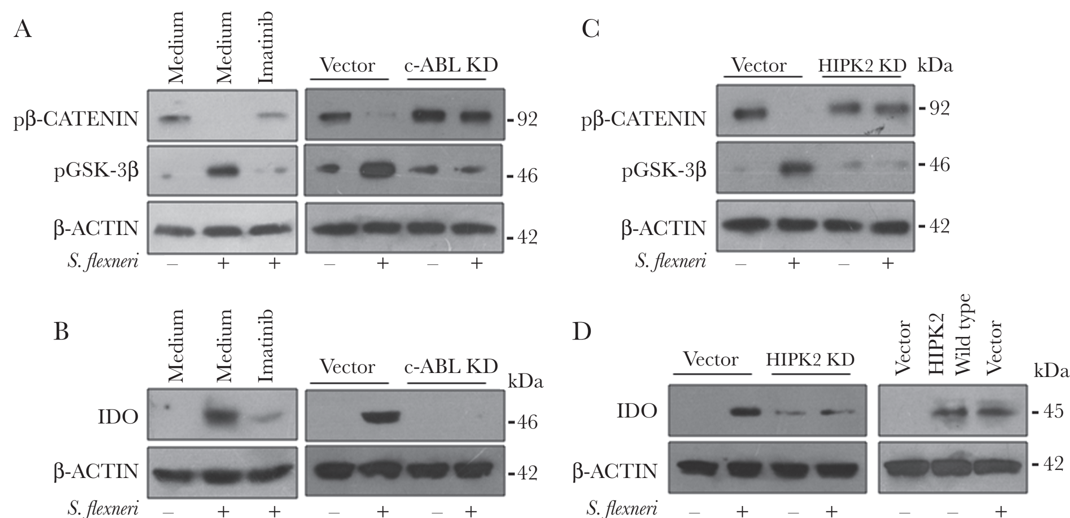

Figure 5. c-Abl mediates indoleamine 2,3-dioxygenase 1 (IDO1) expression driven by epidermal growth factor receptor (EGFR)–WNT through homeodomain interacting protein kinase 2 (HIPK2) serine-threonine kinase. *A* and *B*, Murine peritoneal macrophages (PMs) were pretreated with imatinib (a c-Abl inhibitor; 10 μM) for 1 hour, followed by *S. flexneri* infection for 1 hour, to assess WNT signaling activation (*A*) and for 12 hours to analyze IDO expression by immunoblotting (*B*; *left*). RAW 264.7 macrophages were transfected with c-ABL kinase-dead (KD) construct for 8 hours. Transfected cells were infected with *S. flexneri* for 1 hour to study the status of WNT signaling (*A*) and for 12 hours to assess IDO expression by immunoblotting (*B*; *right*). *C* and *D*, RAW 264.7 macrophages were transfected with HIPK2 KD and HIPK2 wild-type constructs as indicated. Transfected cells were infected with *S. flexneri* for 1 hour to analyze the status of WNT signaling (*C*) and for 12 hours to assess the expression of IDO at the protein level by immunoblotting (*D*). β-actin was used as a loading control. All immunoblots are representative of 3 independent experiments. See also Supplementary Figure 2.

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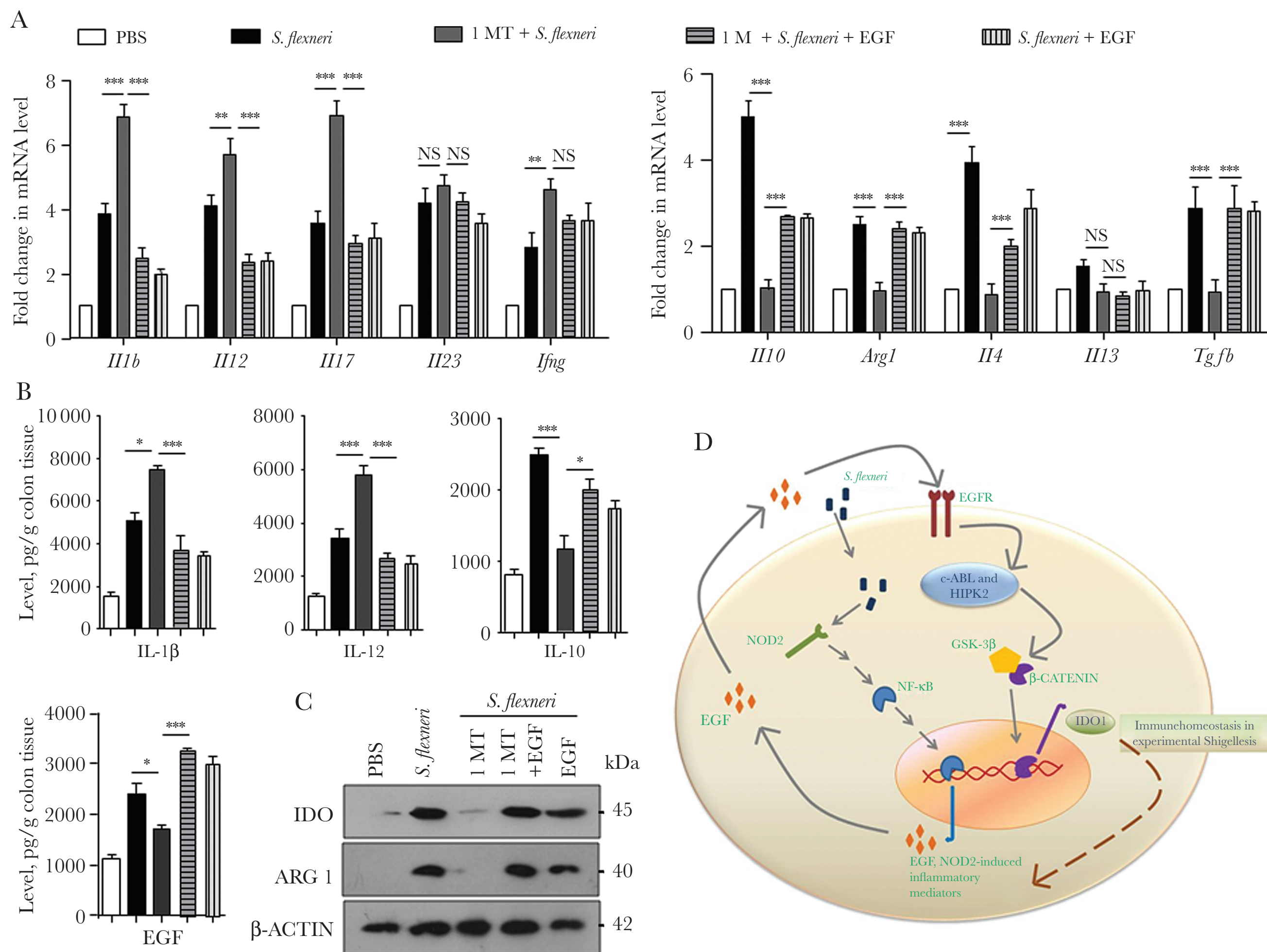

Figure 6. Indoleamine 2,3-dioxygenase 1 (IDO1) maintains homeostasis by modulating the production of inflammatory mediators. Balb/c mice were administered 1-methyl-D-tryptophan (1MT; 10 mg/mouse), an inhibitor of IDO1, intraperitoneally 12 hours before intraperitoneal infection with *Shigella flexneri* (5×10^8 colony-forming units), followed by recombinant murine epidermal growth factor (EGF; 3 µg/mouse intraperitoneally) administration 1 hour and 12 hours after infection. At hour 18 of infection, mice from each of the indicated treatment groups were euthanized to obtain colon tissue specimens. *A*, Colons were mechanically homogenized, and RNA was isolated to assess the differential expression of inflammatory mediators by real-time quantitative polymerase chain reaction (qPCR). *B* and *C*, A colon tissue specimen was homogenized by bead beating. The homogenate obtained was assessed for the indicated inflammatory cytokines and EGF by an enzyme-linked immunosorbent assay (*B*) and for ARG1 and IDO1 expression by immunoblotting (*C*). *D*, Model depicting the molecular network used by host cells to acquire immune equipoise during experimental shigellosis. A minimum of 5 mice were used per experimental group. The immunoblot is representative of 3 independent experiments. β-actin was used as loading control. Real-time qPCR data represent means ± standard errors (n = 3). PBS, phosphate-buffered saline. *$P < .05$, **$P < .005$, and ***$P < .001$ by 1-way analysis of variance followed by the Bonferroni post hoc test.

the inflammatory balance. Indeed, EGF was found to reduce the expression of proinflammatory cytokines, compared with their IDO1-inhibited counterparts, with a concurrent increase in the comparative levels of the antiinflammatory mediators (Figure 6A and 6B). EGF, IDO, and ARG1 were also observed to follow the same trend (Figure 6B and 6C), hence aiding in the reconstitution of a balanced intestinal milieu. Furthermore, a marked reduction in bacterial burden in EGF-treated, 1MT-recipient mice with shigellosis as compared to that in IDO1-inhibited mice was observed. This could be attributed to the functional restitution of goblet cells in the presence of an adequate level of antiinflammatory cytokines. Taken together, findings of this study reveal the therapeutic potential of EGF in containing the exacerbated pathology in experimental shigellosis (Figure 6D).

DISCUSSION

Infections with enteric bacteria account for a large proportion of gastrointestinal dysfunction–related morbidity. In situations of inadequate hygiene and sanitation, particularly in impoverished regions, such infections adopt a vicious cycle involving a dysregulated gut and leading to a persistent enteric pathological state prone to infection, malnutrition, impaired growth, and development in children [41, 42]. These environmental enteropathies have been associated with intestinal barrier disruption, insufficient mucosal immunity, local and systemic inflammation, and, eventually, underresponsiveness to antibiotics and vaccines [43].

The current study focuses on the interaction of the enteric pathogen *S. flexneri* with its host. Persistent *Shigella* infections

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are known to cause malnutrition and nonlinear growth in children [1]. The immune evasion strategies adopted by *S. flexneri* and the emerging antibiotic resistance mechanisms have garnered significant attention to the development of efficacious nonantimicrobial interventions [44]. In this realm, high IDO levels have already been associated with environmental enteropathies for enhancing regulator T-cell development, reducing T-helper cell 17 lineages, and thereby increasing mucosal permeability and bacterial translocation [45]. In line with these findings, our study identifies IDO as a crucial homeostat safeguarding the host from hyperinflammation-driven morbidity during *S. flexneri* and *E. faecalis* infection. As such, the implication of IDO as a direct regulator of inflammatory cytokines had previously been understudied. Moreover, we believe that genetic mechanisms encompassing single-nucleotide polymorphisms disrupting IDO1 or physiological conditions altering IDO1 functions may predispose individuals to fatal pathological consequences. Thus, uncovering the mechanisms adopted by host cells through the EGFR-cABL-HIPK2 axis to activate WNT signaling and subsequently express IDO1 would provide critical insights into the ways of potentiating IDO functions for adjunctive therapy in inflammatory dysfunctions of likely diverse origins, including infection, malnutrition, and related enteropathies. Importantly, to date, defective EGFR has been associated with gastrointestinal toxicity apart from cancers [20]; however, the current study prompts further analysis of EGFR as a potential biomarker for diverse inflammatory syndromes. Our current findings reveal a profound effect of EGF in enforcing host homeostasis by controlling inflammation, and existing literature also indicates the efficacy of EGF in ameliorating ulcerative colitis [46]. However, the exact molecular mechanisms and alternative functions of EGF are yet to be elucidated.

Further, the novel finding of *S. flexneri*–driven NOD2 activation yields distinct avenues for exploring the yet unknown contributions of this intracellular pattern-recognition receptor in shigellosis and associated diseases. Finally, it must be noted that EGFR inhibitors [47] and IDO1 inhibitors [48], alone or in combination, are widely used for cancer treatment. In the light of the current investigation, we not only emphasize the possible therapeutic effects of EGF in rescuing the host from inflammatory dysfunction associated with enteric infections, but also project the relevance of careful titration and targeted delivery of such therapeutic adjuncts to avert secondary complications.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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