# Interferon-driven alterations of the host's amino acid metabolism in the pathogenesis of typhoid fever

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**Enteric fever, caused by *Salmonella enterica* serovar Typhi, is an important public health problem in resource-limited settings and, despite decades of research, human responses to the infection are poorly understood. In 41 healthy adults experimentally infected with wild-type *S.* Typhi, we detected significant cytokine responses within 12 h of bacterial ingestion. These early responses did not correlate with subsequent clinical disease outcomes and likely indicate initial host–pathogen interactions in the gut mucosa. In participants developing enteric fever after oral infection, marked transcriptional and cytokine responses during acute disease reflected dominant type I/II interferon signatures, which were significantly associated with bacteremia. Using a murine and macrophage infection model, we validated the pivotal role of this response in the expression of proteins of the host tryptophan metabolism during *Salmonella* infection. Corresponding alterations in tryptophan catabolites with immunomodulatory properties in serum of participants with typhoid fever confirmed the activity of this pathway, and implicate a central role of host tryptophan metabolism in the pathogenesis of typhoid fever.**

*Salmonella enterica* serovar Typhi (*S.* Typhi), the etiological agent of enteric fever (typhoid fever), is a significant cause of morbidity in many parts of the world with poorly resourced health systems (Crump et al., 2004; Lozano et al., 2012), and among travelers (Jensenius et al., 2013; Leder et al., 2013).

Despite decades of research into typhoid fever pathogenesis, neither the survival strategies of this intracellular bacterium in humans nor the intricacies of protective immune responses have been fully explained. *S.* Typhi invades the gut mucosa soon after ingestion, where it is taken up by macrophages and dendritic cells before transit to local lymph nodes. Within 24 h, a clinically insignificant blood stream infection is thought to disseminate the organism to the reticuloendothelial system. Subsequently, a second, more sustained bacteremia can occur, which is accompanied by the onset of fever and constitutional symptoms (Parry et al., 2002; de Jong et al., 2012). Significant evidence indicates bacterial immunomodulatory capabilities, suggesting *S.* Typhi can effectively evade the host immune system (Wangdi et al., 2012), e.g., by expression of Vi-polysaccharide (Sharma and Qadri, 2004; Jansen et al., 2011) or inhibition of autophagy via mTOR activation (Tattoli et al., 2012). The clinical implications of immune evasion include the observation that multiple episodes of typhoid infection are probably required to induce significant protection against natural infection (Saul et al., 2013).

We know little about when, where, or how the human immune system limits, and then clears, *S.* Typhi infection. Whereas immunological responses to *Salmonella* infection are characterized by IFN signatures (de Jong et al., 2012; Sztein et al., 2014), relatively low concentrations of pyrogenic cytokines, including IL-6, IL1β, and TNF, have been found in patients diagnosed with acute typhoid fever (Keuter et al., 1994). Moreover, functional $CD8^+$ T cells are activated by live oral vaccines and likely play a role in cell-mediated immunity

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Abbreviations used: AhR, aryl hydrocarbon receptor; FC, fold-change; GSEA, gene set enrichment analysis; iPS, induced pluripotent stem; LLD, lower limit of detection; MDTH, molecular distance to health; STm, *Salmonella* Typhimurium; TD, typhoid diagnosis.



The Rockefeller University Press $30.00
J. Exp. Med. 2016 Vol. 213 No. 6 1061–1077
www.jem.org/cgi/doi/10.1084/jem.20151025

(CMI; Salerno-Goncalves et al., 2002; Sztein et al., 2014). Finally, as indicated by the efficacy of the parental Vi-polysaccharide vaccine, antibodies also play a role in the protective host response to *S.* Typhi infection (Klugman et al., 1987). Despite this knowledge, the detailed mechanisms and how the different immunological aspects interact to produce effective immune responses are poorly understood.

Humans are the only known natural host of *S.* Typhi and, consequently, studies to elucidate immunopathogenesis have been compromised by the lack of suitable small animal models. To address this knowledge gap, we have recently established a human infection model of typhoid fever in healthy adult volunteers based on early work (Hornick et al., 1970a; Levine et al., 2001; Waddington et al., 2014). In this study, we describe the longitudinal human host responses from the time of bacterial exposure until overt clinical disease develops, highlighting the potential to interrogate molecular disease pathogenesis using a human challenge model. Using integrative analysis of transcriptional and cytokine profiles, clinical outcome, and metabolite data, we build on previously reported response signatures to gain further insight into the complex disease pathogenesis of typhoid fever. Combining data derived from these analyses with data from macrophage and murine infection models highlight an association of the link between IFN responses and the tryptophan metabolism with clinical typhoid fever, providing novel insights into the immunopathogenesis of *S.* Typhi.

## RESULTS

### Longitudinal blood transcriptome of participants challenged with *S.* Typhi

In this study, we orally challenged 41 healthy adults with *S.* Typhi in sodium bicarbonate solution, as described previously (Waddington et al., 2014); 61% (25/41) of whom were subsequently diagnosed with acute typhoid fever during a 14-d challenge period. At the time of diagnosis, most participants had systemic symptoms, including fever and bacteremia (Waddington et al., 2014). Gene expression profiles were generated based on mRNA purified from whole blood before and at time points after challenge. In the blood of typhoid participants at diagnosis (time point: typhoid diagnosis [TD]), we detected marked differential expression from 24 h before (TD-24) to at least 24 h after diagnosis (TD+24; subsequently termed acute typhoid fever; Table S1). This perturbation involved changes in the relative expression of 2,301–3,450 transcripts (Student's *t* test; Benjamini-Hochberg [BH], $P < 0.05$; FC, ±1.5), which resolved in most participants by 14 d (D14) after challenge (Fig. 1 A).

As a comparative cohort, we analyzed transcriptional profiles from those individuals who did not develop typhoid fever despite oral challenge by measuring responses at 7 (nD7) and 14 d (nD14) after challenge (Fig. 1 A). Although participants who appeared resistant during the challenge period were clinically inconspicuous, we detected moderate differential expression of 335 genes (Student's *t* test; BH, $P < 0.05$; FC, ±1.5) 7 d after challenge. Differential expression of these genes was indistinguishable from pre-challenge levels by nD14 after challenge in these participants. Unsupervised clustering highlighted the significant differences between samples derived from participants with acute typhoid and the remaining samples (Fig. 1 A). Comparison of weighted molecular distance to health (MDTH) scores, a measure of transcriptional perturbation (Pankla et al., 2009), identified significant differences in transcriptional perturbation between samples taken during acute disease and samples taken at nD7 from participants who did not develop typhoid (Fig. 1 B).

### IFN, cell cycle, and aryl hydrocarbon receptor signatures are differentially expressed during acute disease

To interpret the transcriptional changes observed after challenge, we searched our data for pathways and transcription factor (TF) binding sites over-represented in participants diagnosed with typhoid fever using the analysis tool InnateDB (Lynn et al., 2010). We observed a striking pattern of pathways representing type I and type II IFN signaling (Fig. 1 C). Furthermore we observed a molecular signature of TFs associated with cell cycle control, including E2F complexes, DP-1, and Myc, in addition to those mediating IFN responses. We also detected overrepresentation of the TF aryl hydrocarbon receptor (AhR), previously shown to play a role in tumor biology (Opitz et al., 2011; Fig. 1, D–F).

Next, we compared our data to the transcriptome derived from patients with blood culture–confirmed typhoid fever in Vietnam ($n = 38$), a region endemic for enteric fever (Thompson et al., 2009). Although we observed considerable heterogeneity between the Vietnam study and the challenge model at the gene level (33.3% overlap; Fig. S1 A), gene set enrichment analysis (GSEA) determined significant enrichment of differentially expressed genes of the Vietnam data in the acute typhoid signature (Fig. S1 B). Moreover, similarities between the transcriptional responses to typhoid in the two-study cohorts were supported when the data were analyzed at the pathway level (Fig. S1 C).

### Modular analysis of the blood transcriptome confirms a dominant IFN signature during acute typhoid infection

To gain a more detailed insight into the nature of the transcriptional response after challenge with *S.* Typhi, we mapped these data to a conceptual framework consisting of 62 predefined transcriptional modules, as previously described (Chaussabel et al., 2008; Chaussabel and Baldwin, 2014).

Transcriptional dynamics during acute typhoid demonstrated a significant relative overexpression of all three IFN modules (M1.2, M3.4, and M5.12). Furthermore, monocyte (M4.14), inflammatory (M4.6 and M5.1), and innate immune modules (M3.2, M4.2, and M4.13) were also relatively overexpressed. In addition, we observed a moderate relative overexpression of neutrophil-related genes (M5.15) and significant activation of apoptosis-related (M6.6 and M6.13) and

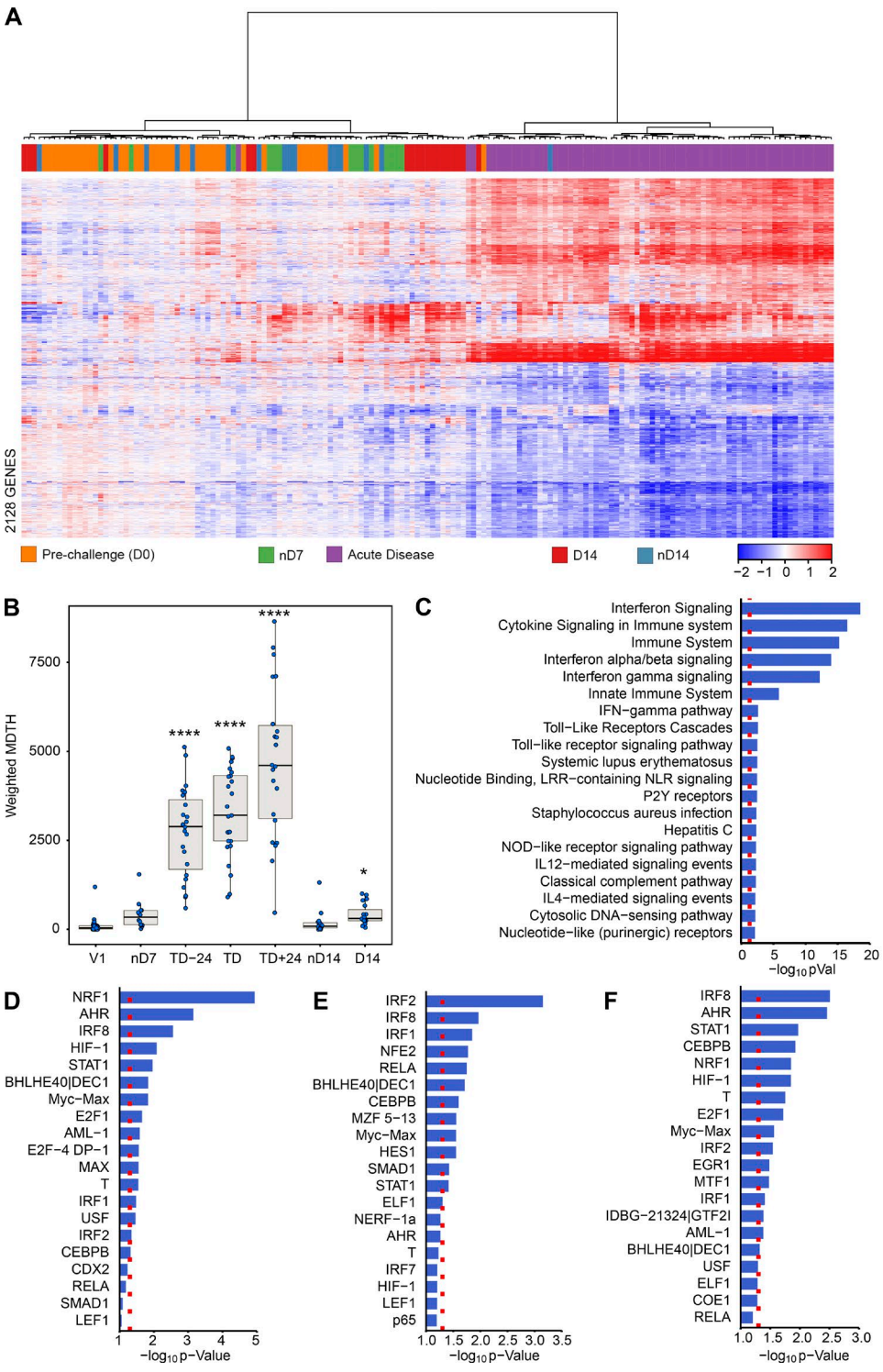


Figure 1. **Transcriptional signatures associated with acute typhoid fever.** (A) Unsupervised clustering of genes differentially expressed in samples derived in participants diagnosed or not diagnosed with typhoid after challenge. Color bar represents time points: nD7 ($n$ = 14, green) and nD14 ($n$ = 15, blue) in participants not diagnosed with typhoid; TD-24 h ($n$ = 23), TD ($n$ = 25), and TD+24 h ($n$ = 22; acute disease, purple) and D14 ($n$ = 19; red) in diagnosed participants. (B) MDTH scores based on the expression of the genes displayed in A. *, P < 0.05; ****, P < 0.0001 compared with challenge baselines (D0) using Kruskal-Wallis test with Dunn's correction for multiple testing. (C) Top 20 most significant pathways overrepresented at TD ($n$ = 25). (D–F) Top 20 most significantly differentially expressed transcription factors at TD-24 h ($n$ = 23; D), TD ($n$ = 25; E), and TD+24 h ($n$ = 22; F; red dotted line, P = 0.05). Euclidean distance clustering using the ward algorithm (A). Data are median with 25th/75th percentile (B).

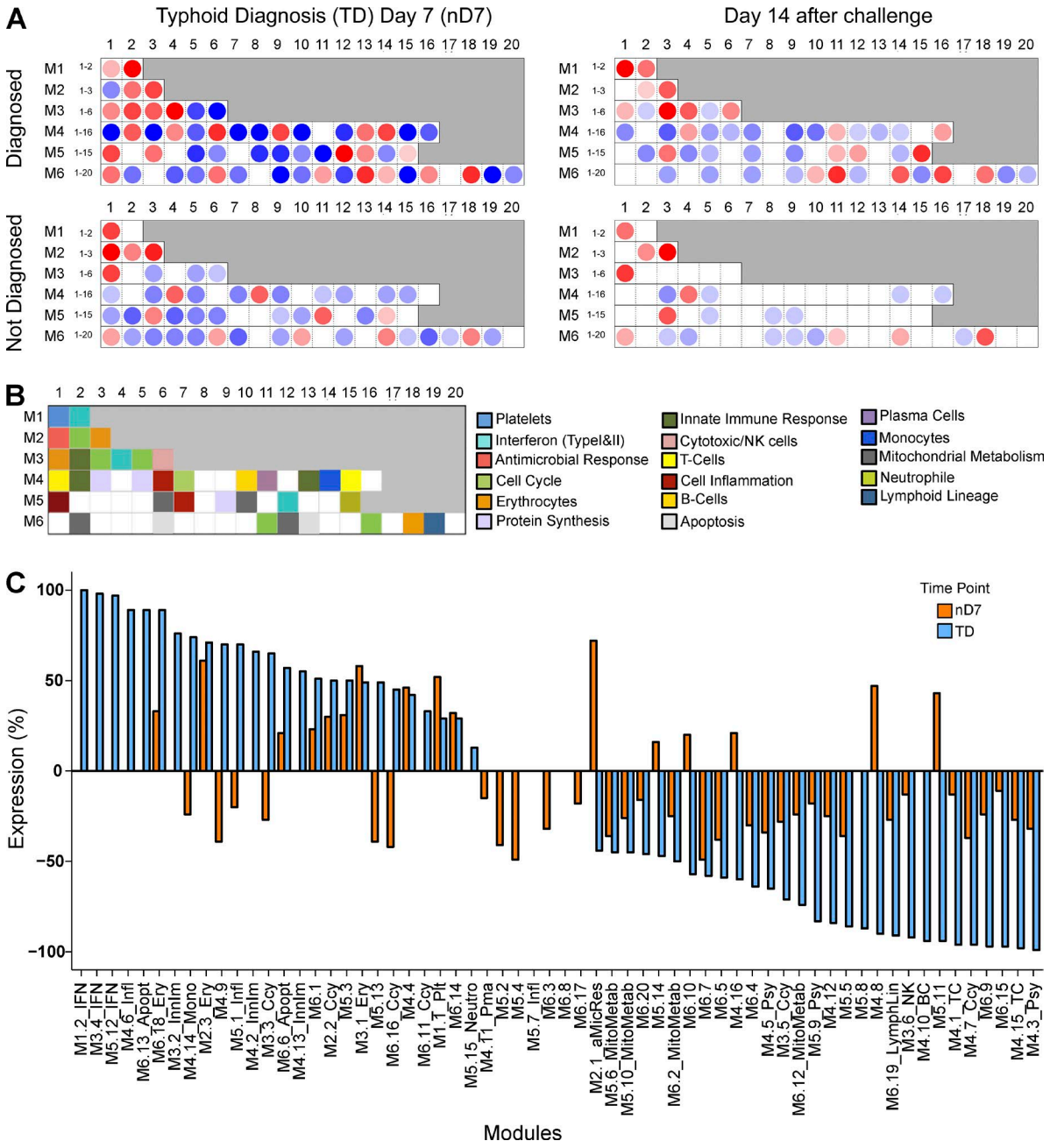


Figure 2. **Modular analysis of the acute typhoid transcriptome.** (A) Modular maps were constructed at each time point separately as described in Materials and methods. Red or blue coloration indicates the proportion of over- or under-expressed transcripts ($P < 0.05$, unpaired Student's *t* test) contained in each module, respectively (white, not expressed; blue, underexpressed; red, overexpressed). (top) Diagnosed participants at TD ($n = 25$) and D14 ($n = 19$); (bottom) participants who stayed well at nD7 ($n = 14$) and nD14 ($n = 15$). (B) Modular map annotation key. (C) Modular expression (in % significant genes) of each module was plotted ordered according to expression (positive to negative) at time of diagnosis (TD).

cell cycle modules (M2.2, M3.3, M6.11, and M6.16; Fig. 2, A and B). In contrast, whereas modules do not reflect cell types directly, modules representing genes related to signaling and stimulation of B and T cells (M4.1, M4.10, and M4.15), the lymphoid lineage (M6.19) and NK cells (M3.6) were relatively underexpressed during acute typhoid fever (Fig. 2, A and B) as were modules representing mitochondrial metabolism and protein synthesis.

Several differences in transcriptional activity were observed at nD7 when compared with those diagnosed with disease. Notably, whereas modules relating to IFN activity were not perturbed, those representing monocyte activation,

inflammation, and the antimicrobial response, and some modules representing cell cycle genes, were inversely expressed compared with responses seen in participants with acute typhoid fever (Fig. 2 C).

### Humans respond to *S.* Typhi within hours of ingestion

We previously showed that individuals who did not develop typhoid fever despite oral challenge failed to mount a significant anti–*S.* Typhi serological response (Waddington et al., 2014). This raised the question of whether these participants experienced any significant host–*S.* Typhi interaction after ingestion, or whether challenge simply failed, perhaps as a result of bacterial destruction by gastric acid. Consequently, we determined the longitudinal plasma cytokine response during the 14-d challenge period in all study participants (Tables S2 and S3). Interestingly, we observed a significant induction of plasma cytokines, including sCD40L, fractalkine ($CX_3CL1$), GROα, IL1RA, EGF, and VEGF, in the majority of participants 12 h after challenge (Fig. 3 A and Table S4). The magnitude of this signal was dose dependent, had disappeared by 24 h after challenge, and was not associated with subsequent development of typhoid fever (binary logistic regression and Cox proportional hazard models; Tables S5, S6, and S7).

To corroborate that the early cytokine signature was not associated with sodium bicarbonate ($NaHCO_3$) used to neutralize stomach acid before challenge, we performed a substudy in which healthy adult volunteers ($n = 10$) were given 120 ml $NaHCO_3$, omitting the bacterial suspension. Plasma cytokines were measured before and at 12 and 24 h after ingestion. $NaHCO_3$ failed to induce any induction of measured cytokines (Fig. 3 B), indicating that these data represent a true early response signal attributable to *S.* Typhi ingestion.

### Cytokine responses confirm blood transcriptome signatures during acute typhoid fever

As transcriptional responses were dominated by IFN-signatures known to be associated with the intracellular pathogen *S.* Typhi (Thompson et al., 2009), we expected significant up-regulation of cytokines related to the IFN response. The peripheral blood cytokine profile of participants with acute typhoid fever was indeed dominated by IFN-γ related (IFN-γ and IP-10) and inflammatory cytokines such as TNF (Fig. 4, A–C; and Fig. S2, A–D). In contrast, volunteers who stayed well after challenge demonstrated no significant responses during the challenge period after disappearance of the early 12-h cytokine signal (Fig. 4, D–F; and Fig. S2, E–H). Whereas these data mirror the transcriptional profile, they confirm previous findings from the field highlighting the sustained IFN-γ response during acute typhoid fever.

### Modules associated with acute typhoid fever correlate with bacteremia and humoral responses in participants diagnosed with typhoid fever

A significant advantage of the human challenge model is the variety of study parameters available to relate molecular events with phenotypic outcomes. To investigate these relationships, we correlated weighted MDTH scores with markers of clinical disease and microbiological status in participants diagnosed with typhoid fever. MDTH scores were positively associated with maximal temperature change from baseline ($\Delta Temperature_{max}$), maximum CRP concentration ($CRP_{max}$), and duration of bacteremia (hours), but negatively associated with time-to-diagnosis (Fig. 5, A–D). As the magnitude of transcriptional perturbation was closely related to the clinical phenotype, we next aimed to dissect the transcriptome to explore potential relationships between specific gene expression patterns and outcome parameters. Correlation of modular expression (Obermoser et al., 2013) at TD with outcomes related to typhoid fever (including humoral responses and bacteremia; Waddington et al., 2014), identified a significant association between IFN-related gene overexpression and duration of positive blood culture, implying a direct relationship between bacteremia and enhanced IFN response (Fig. 5 E). Modules representing general innate immune and inflammatory responses (IFN, inflammation, innate immunity, and apoptosis) were also positively associated with clinical markers of severity (including CRP levels and temperature), but negatively associated with time-to-diagnosis. Furthermore, we observed significant correlations of several cell cycle modules with antibody levels at day 28 after challenge. Importantly, these modules were relatively underexpressed in participants who stayed well after challenge, a population that importantly failed to produce any notable anti–*S.* Typhi antibody responses, confirming that this signature appears to predict subsequent antibody responses to *S.* Typhi (Fig. 5 E).

### Induction of the IFN–tryptophan metabolism interactome after challenge with *S.* Typhi

Although IFN responses during *Salmonella* infection are well described, are thought to originate from T and NK cells (Salerno-Goncalves et al., 2002), and are associated with intracellular pathogens, this response is poorly understood, particularly in humans. Closer interrogation of the genes related to the observed IFN signature revealed many genes belonging to the guanylate-binding protein (GBP) family (previously linked to *Salmonella* infection; Meunier et al., 2014), and genes linking IFN-γ responses with the tryptophan metabolism, including *IDO1* (indoleamine 2,3-dioxygenase 1). This rate limiting enzyme in the tryptophan catabolism (Munn and Mellor, 2013) is co-regulated by the transcription factors STAT1 and IRF1 (Chon et al., 1996), and its activation leads to direct stimulation of the transcription factor AhR (Opitz et al., 2011), all three of which are over-represented during acute typhoid fever (Fig. 1, D–F). To visualize this molecular pathway, we custom curated the interactions between IFN-γ signaling, IDO1, and AhR using InnateDB and Cytoscape (Shannon et al., 2003; Lynn et al., 2010; "IFN–tryptophan interactome"). Superimposing this interactome with mean gene expression data after challenge indicated a profound differential regulation of these genes during acute typhoid fever,

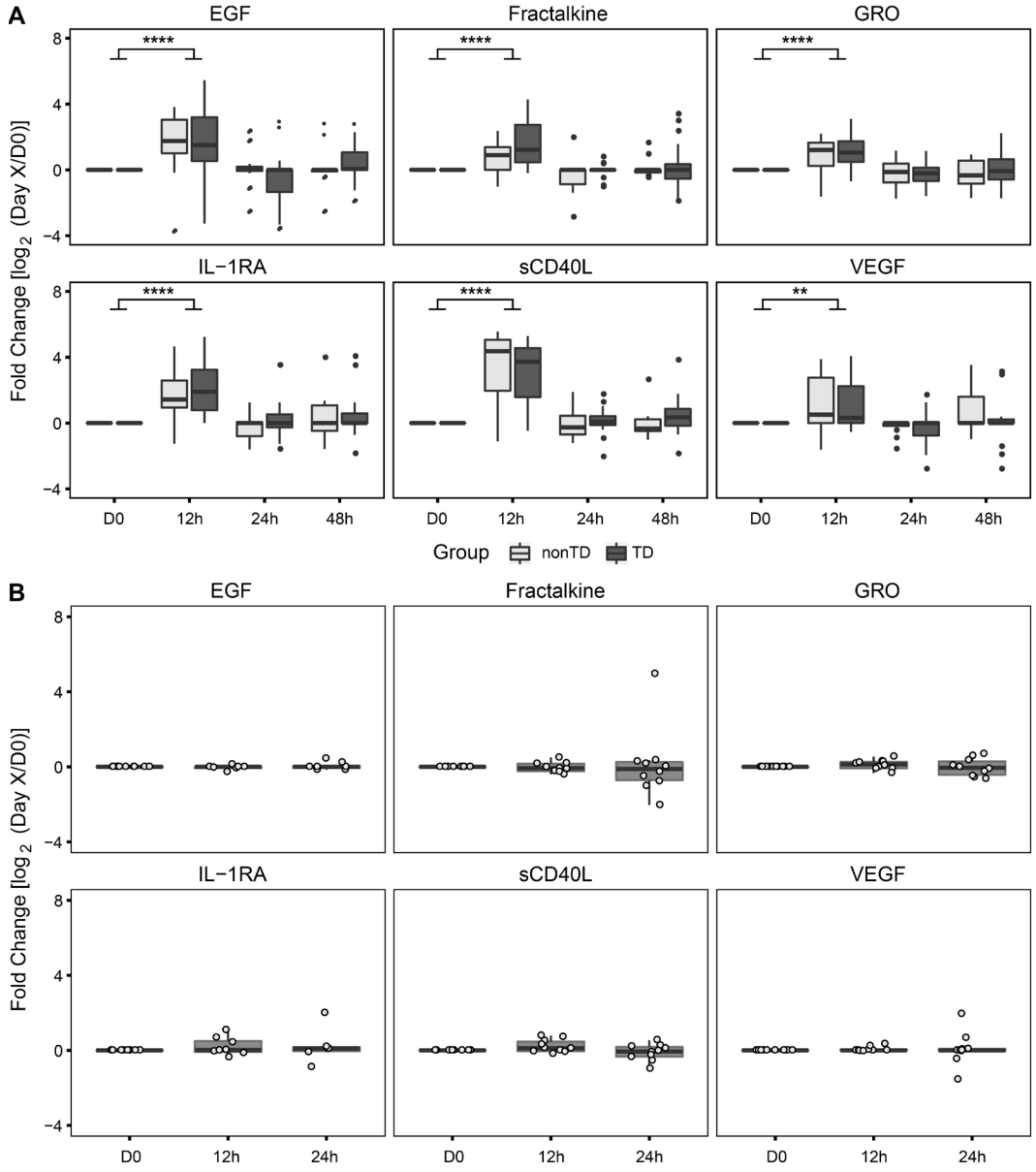


Figure 3. **Plasma cytokine profiles within hours after challenge.** Cytokine induction [$\log_2$ FC/D0] at prechallenge (D0), 12, 24, and 48 h after challenge in participants who were diagnosed ($n$ = 25, dark gray) or stayed well ($n$ = 16, light gray) after challenge with *S.* Typhi (A). Cytokine induction [$\log_2$ FC/D0] at 12 h, 24 h after ingestion of sodium bicarbonate solution ($n$ = 10; B). Data are median with 25th/75th percentile and run in duplicates. **, P < 0.01; ****, P < 0.0001; using unpaired Student's *t* test (Table S4).

but not in those who stayed well after oral challenge (Fig. 6, A and B). Differential expression of key genes was confirmed by performing quantitative PCR on a subset of samples (Fig. S3). These data suggest a link between *S.* Typhi–associated IFN responses and the tryptophan catabolism.

### The tryptophan catabolism is associated with bacteremia and clinical disease

We next sought to validate the activation of the tryptophan catabolism pathway by measuring key metabolites (tryptophan, kynurenine, and quinolate) in serum from challenge study participants. Kynurenine and quinolate levels were significantly increased at TD compared with prechallenge in those participants diagnosed with typhoid (P = 0.0001 and P < 0.0001, respectively). Whereas tryptophan was not significantly decreased (P = 0.06; Fig. 6 C), none of these metabolites were changed at nD7 in those participants who stayed well after challenge (Fig. 6 D). Finally, linking these changes to disease outcome, key components of this pathway, including IFN-γ, kynurenine, and quinolate changes,

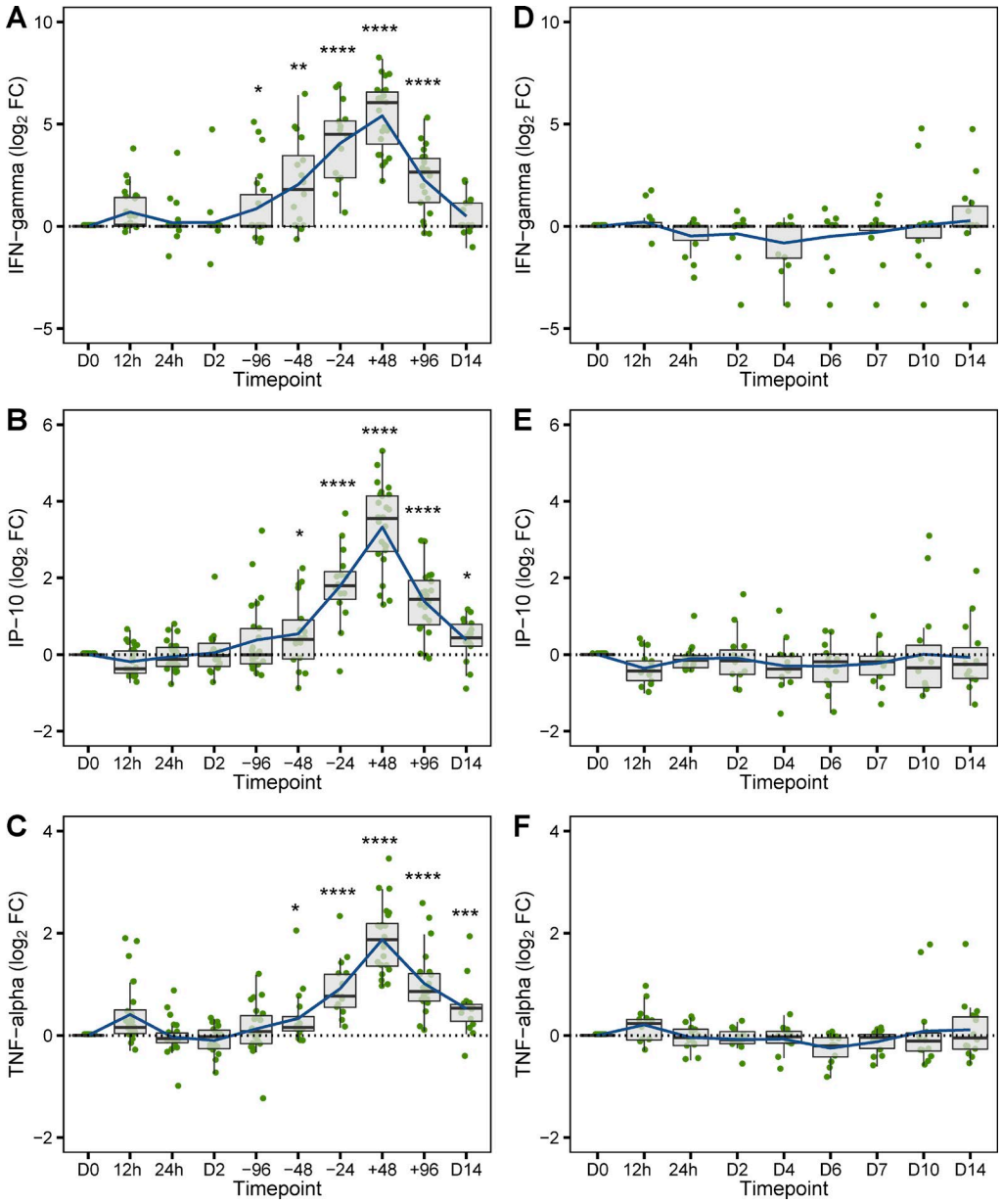


Figure 4. **Longitudinal plasma cytokine profiles of participants challenged with *S.* Typhi.** Longitudinal cytokine response profiles [$\log_2$ FC/D0] for participants developing typhoid fever (A–C, $n = 25$) and those who were not diagnosed after challenge (D–F, $n = 16$). Data are median with 25th/75th percentile, and blue line depicts the mean. Each sample was run in duplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ using one-sample Student's *t* test.

were significantly correlated with the same microbiological (bacteremia) and clinical ($\Delta Temperature_{max}$) outcome parameters as observed for transcriptional IFN responses (Fig. 6 E). These results strongly suggest that activation of tryptophan catabolism is associated with the clinical disease phenotype of acute typhoid fever.

### Expression of key genes mediating tryptophan catabolism during *Salmonella* infection in vivo

As it is impossible to investigate tissue-specific effects in the human challenge model, we sought to determine whether *Salmonella* infection induces key genes in the IFN–tryptophan signaling axis in a murine model. Thus, we orally challenged C57BL/6N mice with *S.* Typhimurium SL1344 or saline (controls, $n = 5$) and harvested cecum, liver, and spleen 3 d ($n = 4$) and 7 d ($n = 6$) after infection. Marked induction of target genes involved in the IFN–tryptophan signaling was observed in most tissues, with the cecum and the liver showing the most consistent induction (Fig. 7 A). *IDO1* was significantly expressed in the cecum at day 7 only, suggesting a time-dependent expression of these markers in vivo. To link the induction of these genes to IFN-γ signaling specifically,

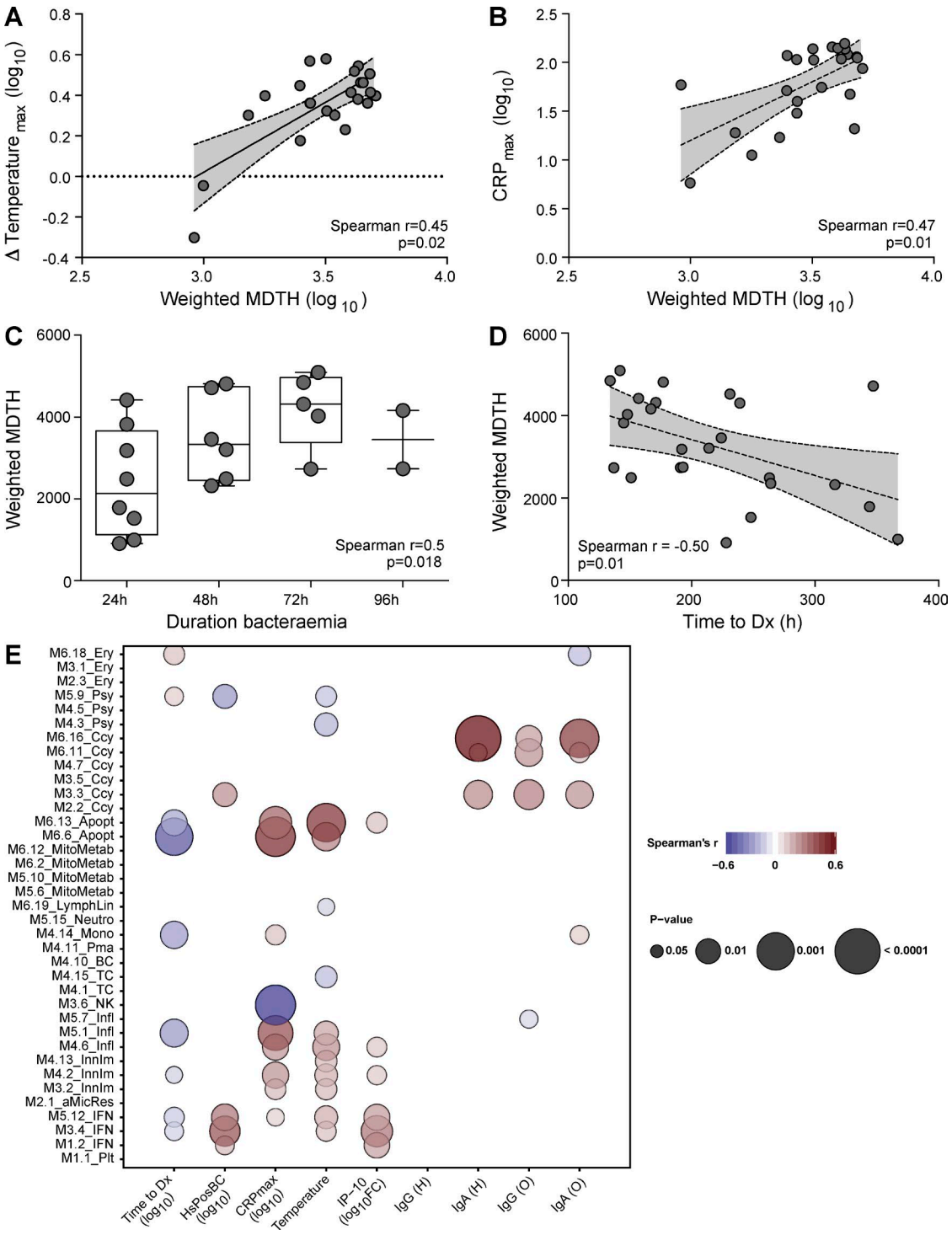


Figure 5. **Relationships among the blood transcriptome to clinically relevant outcomes.** Spearman rank's correlation analysis of weighted MDTH scores with (A) ΔTemperature (maximum change from baseline, $n = 24$), (B) maximum CRP ($n = 24$), (C) duration of bacteremia (in hours; $n = 21$), and (D) time-to-diagnosis (in hours; $n = 25$) in participants diagnosed with acute typhoid fever. (E) Modular expression ($log_{10}$ FC) was calculated for each participant and correlated (Spearman's rank correlation) with clinical and immunobiological outcome measures. Red, positive correlation; blue, negative correlation; bubble size represents correlation p-values.

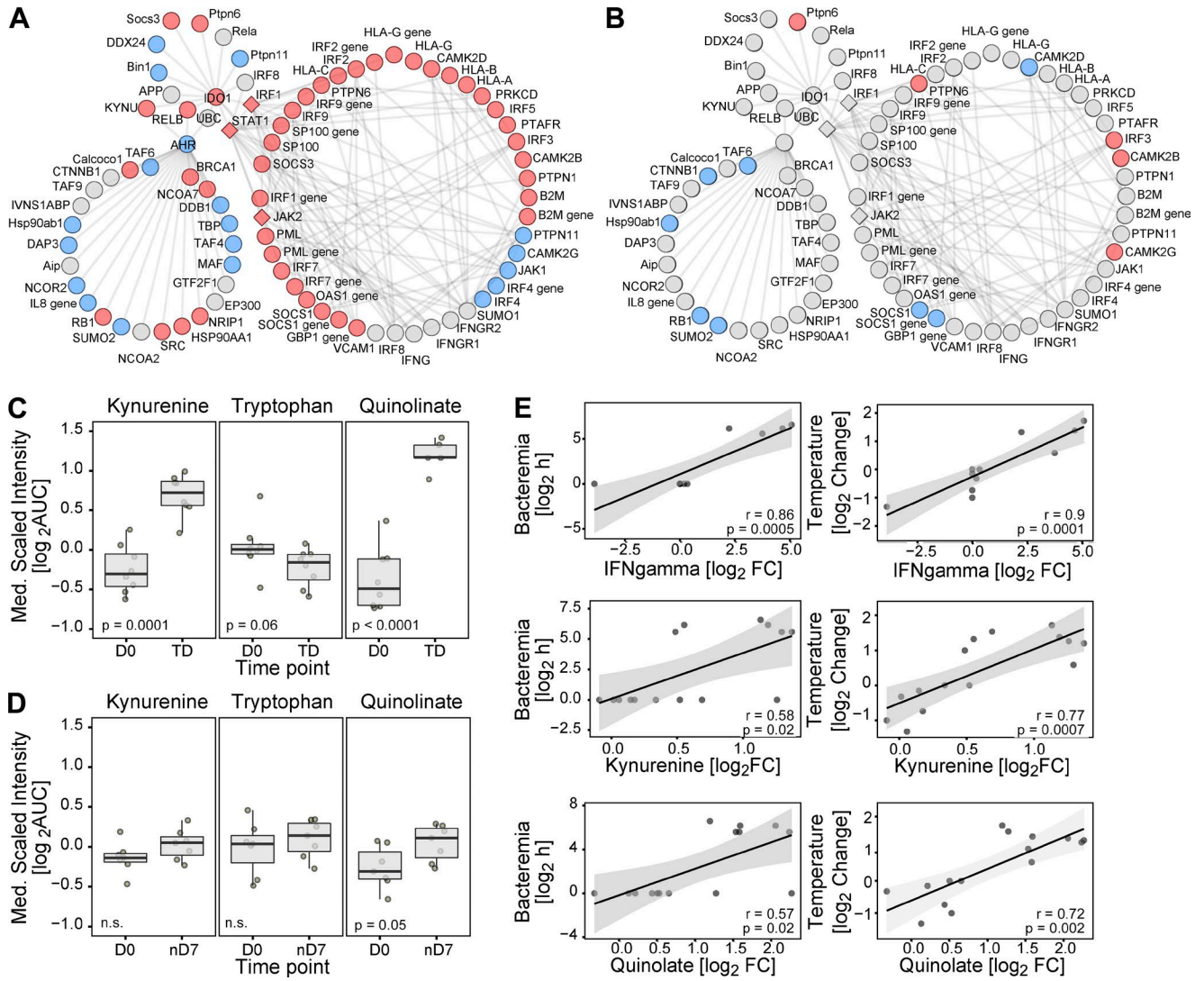


Figure 6. **IFN–tryptophan interactome and metabolites after challenge.** (A and B) Custom IFN–tryptophan metabolism interactome superimposed with the gene expression during acute disease (mean expression of TD-24 h [$n$ = 23], TD [$n$ = 25], and TD+24 h [$n$ = 22]; A) and day 7 after challenge in those not diagnosed after challenge (nD7; $n$ = 16; B). Kynurenine, tryptophan, and quinolinate changes from prechallenge control samples in participants diagnosed with typhoid (TD; $n$ = 8; C) and those who stayed well (nD7; $n$ = 7; D). (E) Spearman's rank correlation of microbiological (bacteremia in hours) and clinical outcome parameters ($\Delta$Temperature$_{max}$) with IFN-γ (plasma cytokine; $n$ = 10–11), kynurenine ($n$ = 15), and quinolate ($n$ = 15). Data are mean and 25th/75th percentile and samples were run in duplicates. Statistical differences were determined using paired Student's $t$ test (C and D).

we infected IFN-γR KO mice ($n$ = 6) and WT mice ($n$ = 6) with *S.* Typhimurium SL1344 and monitored expression of *IRF1*, *STAT1*, *IDO1*, *KYNU*, and *WARS* once animals lost 12% of their baseline body weight. All genes except *KYNU* were significantly down-regulated in IFN-γR KO but not WT mice (Fig. 7 B), confirming a critical role of IFN-γ in the induction of these genes in tissues typically infected by *Salmonella* after oral challenge.

### *Salmonella* alone fails to induce key molecules of the tryptophan catabolism

Because macrophages are a primary target for *Salmonella* invasion and IFN-γ, we sought to test whether the IFN-γ–tryptophan signaling axis is induced in macrophages after stimulation with two *Salmonella* serovars (Typhi and Typhimurium). To this end, we extended the gene list of the interactome in Fig. 6 to capture the broader IFN-γ signaling and tryptophan metabolism and validated its differential expression after human challenge (Fig. 7 C). Using a gentamicin protection assay (van Wilgenburg et al., 2013), protein abundances corresponding to this gene list measured in IFN-γ–primed macrophages after infection with *S.* Typhimurium showed a distinct clustering behavior compared with controls, seemingly driven by the IFN-γ priming of macrophages (Fig. 7 C, inset). Specifically, key genes and proteins of the IFN-γ–tryptophan signaling axis were markedly up-regulated

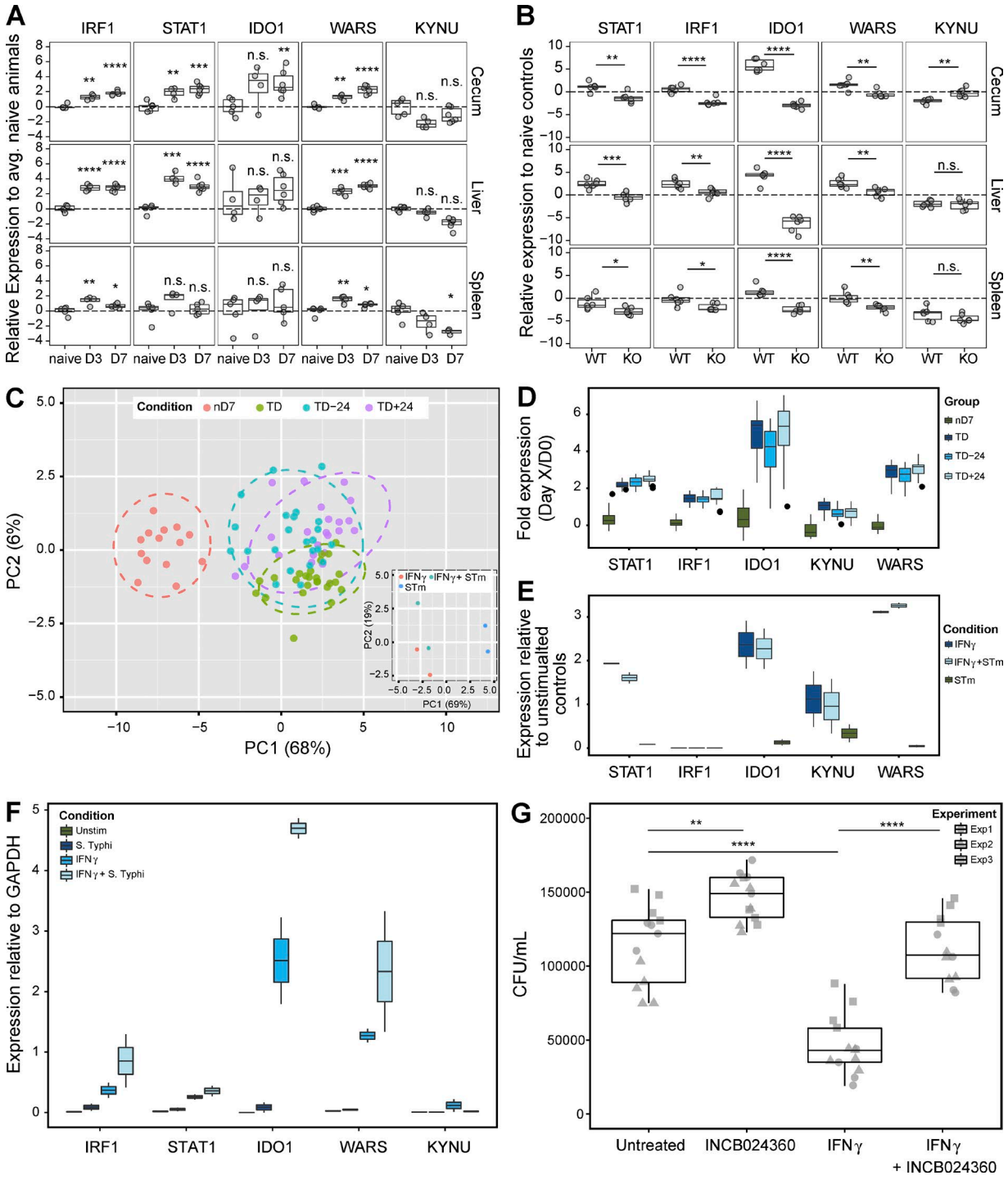


Figure 7. **IFNγ-mediated IDO1 expression in vivo.** (A) Gene expression was measured in spleen, cecum, and liver of WT mice at D0 ($n$ = 5), day 3 ($n$ = 4), and day 7 ($n$ = 6) after infection. (B) Gene expression was measured in spleen, cecum, and liver when WT ($n$ = 6) and IFN-γR$^{-/-}$ ($n$ = 6) mice experiencing 12% weight loss PI. (C) Gene expression ($\log_2$ FC) of the IFN–tryptophan signaling network genes at day 7 ($n$ = 16) and TD−24 h ($n$ = 23), TD ($n$ = 25), and TD+24 h ($n$ = 22) in those who stayed well or were diagnosed after infection. (C, inset) Protein expression ($\log_2$ FC) in IFN-γ–primed macrophages alone (IFN; two independent experiments), infected with *S.* Typhimurium (IFN+STm; two independent experiments), or unprimed macrophages infected with *Salmonella* (STm; two independent experiments). Target gene (D) or protein (E) expression ($\log_2$ FC) in whole blood of participants challenged with *S.* Typhi (nD7, $n$ = 16; TD-24, $n$ = 23; TD, $n$ = 25; TD+24, $n$ = 22) and macrophages stimulated with *S.* Typhimurium (two independent experiments), respectively. (F) Gene expression ($\log_2$ FC) of macrophages alone (unstim), infected with S. Typhi BRD948 (*S.* Typhi), IFN-γ–primed macrophages (IFN-γ), and INF-γ–primed and *S.* Typhi infected (IFNγ+BRD; two independent experiments/condition). (G) Unprimed macrophages were infected with *S.* Typhi BRD and IDO1 inhibitor alone

in participants diagnosed after challenge in IFN-γ–primed macrophages in the presence of *S.* Typhimurium (Fig. 7, D and E). In addition, we complemented these results with stimulation of macrophages with *S.* Typhi, validating the profound induction of the key genes in this pathway after IFN-γ priming (Fig. 7 F). Interestingly, a less profound induction of target genes and proteins was observed when macrophages were stimulated with *Salmonella* alone, indicating that the microenvironment, including the presence of IFN-γ, is important in the regulation of this complex signaling network. Finally, to shed light on the impact of this signaling pathway on bacterial killing by macrophages, we monitored bacterial survival in IFN-γ–primed and unprimed macrophages with or without the IDO1 inhibitor INCB024360 (Liu et al., 2010). Whereas IFN-γ–primed macrophages were capable of killing *S.* Typhi, preincubation of macrophages with INCB024360 significantly impaired bacterial killing. Interestingly, in unprimed macrophages, treatment with the inhibitor increased survival of intracellular *Salmonella* (Fig. 7 G), potentially due to activation of the IDO1 pathway by stimulation of innate immune receptors (Salazar et al., 2015). Collectively, these data suggest that IFN-γ induces key genes of the IFN-γ–tryptophan pathway in macrophages infected with two different *Salmonella* serovars, and selective inhibition of IDO1 reduces the ability of these cells to contain bacterial growth.

## DISCUSSION

Infection with *S.* Typhi affects the lives of over 20 million people worldwide each year, yet understanding of human disease pathogenesis remains limited (Hornick et al., 1970a,b). For the first time in over 40 yr, we used a highly controlled infection model to study, in detail, human responses to *S.* Typhi, and relate these responses to the clinical and immunological phenotype. Using integrative analysis of multifaceted data, we describe a link between IFN responses and changes in metabolites, highlighting for the first time the major role of host tryptophan metabolism in the immunopathogenesis and clinical phenotype of typhoid fever.

Identifying early responses to infection with *Salmonella* has, to date, not been possible in the field setting. In our study, we observed significant induction of peripheral blood cytokine production within the first 24 h after exposure, a time frame consistent with bacterial invasion of small bowel mucosal tissue. The association of early cytokine induction with the dose of bacterial exposure and the lack of correlation with subsequent diagnosis suggests that this response originates from the initial contact of *S.* Typhi with the intestinal mucosa. Studies describing some of these cytokines as markers of intestinal inflammation and mucosal responses during inflammatory bowel disease (Beck and Podolsky, 1999; Danese et al., 2003) support these findings. These data give the first description of an early systemic cytokine response to infection with *S.* Typhi in humans.

In this study, we used whole-blood gene expression studies to interrogate in detail the human host responses of volunteers experimentally infected with *S.* Typhi. Although we detected profound changes in gene expression during acute disease, participants who were not diagnosed also showed mild perturbation of gene expression 7 d after challenge. Notably, reanalysis of a published dataset highlighted marked similarities between experimentally challenged participants and culture-confirmed typhoid fever patients in a field setting, indicating the direct relevance of our results to real-world typhoid in endemic settings (Thompson et al., 2009).

To gain insights into immune responses to acute typhoid fever, we dissected the transcriptome using integrative analysis and correlated gene expression patterns with specific clinical and immunobiological outcomes. Our data indicated a profound positive association between microbiological outcomes and differential gene expression, suggesting that persistence of clinically relevant bacteremia drives much of the transcriptional change observed. Further dissection of the transcriptome indicated a significant relationship between cell cycle signatures and humoral responses 28 d after challenge. Interestingly, these gene signatures were underexpressed in participants who stayed well after challenge, a population who did not exhibit any significant humoral responses. Activation of cell cycle signatures are associated with antibody responses after influenza vaccination (Bucasas et al., 2011) and may be explained by a proliferative boost of antigen-specific B cells in germinal centers (Gitlin et al., 2014). These findings are reflected in our transcriptome analysis, suggesting that this relationship holds true in real life infection with *S.* Typhi in humans.

The use of this challenge model enabled us to interrogate multiple facets of the host response to gain more detailed insight into the pathogenesis of typhoid fever. We observed a sustained IFN-related gene signature during acute typhoid disease in samples derived from the field and human challenge model, which was absent in participants who were not diagnosed after challenge. These data were confirmed at the cytokine level, likely reflecting the intracellular lifestyle of *S.* Typhi, driving an IFN-related phenotype associated with typhoid fever (Hess et al., 1996). Whereas IFN-γ responses have been associated with *Salmonella* infections (Lammas et al., 2000; MacLennan et al., 2004), we observed the overexpression of genes linking IFN-γ signaling with the tryptophan catabolism, mediated by IDO1. Capitalizing on the

(INCB024360), after IFN-γ–priming (INF-γ), after IFN-γ priming and IDO1 inhibition (IFNγ+INCB024360) or alone (untreated), and bacterial counts were determined (three independent experiments/condition). At least three mice per group and time point were analyzed (A and B). Circles represent 95% CI (C). Data are mean FC over appropriate controls with 25th/75th percentile (A, B, and D–G). At least two independent experiments (C, inset, and E–G) with at least two biological repeats were performed. Q-PCRs were run in duplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ using unpaired Student's *t* tests.

unique availability of serum samples collected after challenge, we observed significant alterations of metabolite levels downstream of IDO1. These were associated with bacteremia and clinical parameters, strongly suggesting activation of this pathway and an involvement in disease pathogenesis. Intriguingly, *Salmonella* alone was unable to induce key molecules of this signaling pathway in macrophages unless exogenous IFN-γ was provided, suggesting an important role of IFN-γ in this event. Using a well-established murine model for *Salmonella* infection, we confirmed the pivotal role of IFN-γ in the induction of genes involved in the tryptophan metabolism after *Salmonella* infection in vivo. Notably, the first enzyme downstream of IDO1, KYNU, seemed to be inversely regulated in mice, indicating a more complex regulatory mechanism during infection.

Intriguingly, work from the last century reported the excretion of metabolites of the kynurenine pathway were detected in the urine of patients with typhoid fever before the disease was recognized to be caused by *Salmonella* (Rapoport and Beisel, 1971). Subsequently, IDO1 and the tryptophan metabolism have been implicated in the response to infection with several pathogens (Yoshida et al., 1979; Fujigaki et al., 2002; Silva et al., 2002; Peng and Monack, 2010; Blumenthal et al., 2012; Weiner et al., 2012; Niño-Castro et al., 2014), possibly to starve intracellular pathogens of their nutrients (Zhang and Rubin, 2013). Intriguingly, although IFN-γ–primed macrophages effectively contain intracellular replication of *S.* Typhi, inhibition of IDO1 abrogated this effect. These data provide evidence that IDO1 may be involved in controlling intracellular *Salmonella* growth, possibly through decreasing tryptophan levels. Conversely, the consequent increase in kynurenine may modulate protective immune responses in vivo by activation of regulatory T cells, and inhibition of T cell proliferation (Munn et al., 1999; Sharma et al., 2007; Zelante et al., 2009), thus resulting in immunomodulatory effects. Indeed, we observed increased expression of gut-homing (α4β7 and CXCR3) and activation markers (PD-1) by regulatory T cells in PBMCs from participants during acute disease (compared with participants not diagnosed with typhoid) using an in vitro T cell stimulation assay (McArthur et al., 2015). Although more detailed experiments are warranted, our data suggest an underappreciated role of IDO1 activation and the host's metabolism in the host response to *Salmonella* infection.

Collectively, we demonstrated the usefulness of this unique model in investigating disease pathogenesis. Although the human challenge model limits us in providing definite proof of some mechanistic aspects, our data suggest important, novel aspects of *S.* Typhi infection, including previously unknown early responses to infection and an unappreciated role of IDO1 in the host response to *Salmonella* infection.

## MATERIALS AND METHODS

**Study design and participants.** The present data were derived from clinical samples collected during the development of a human challenge model of typhoid fever using microbial dose escalation after obtaining informed consent (UKCRN ID 9297; approval: Oxfordshire Research Ethics Committee A [10/H604/53]) as described previously (Waddington et al., 2014). We used these samples to interrogate the host response to typhoid fever in a highly controlled experimental setting. Healthy, infection/vaccine-naive adult volunteers (participants; $n = 41$; age range, 19.4–46.5 yr) were challenged with a single oral dose of virulent *S.* Typhi (Quailes strain) bacteria suspended in 30 ml sodium bicarbonate solution [17.5 mg/ml], and after pretreatment with 120 ml sodium bicarbonate solution to neutralize gastric acid. Oral challenge inocula were either low dose ($n = 21$; median [range] = $1.3 \times 10^3$ CFU [$0.7 \times 10^3 – 1.8 \times 10^3$]) or high dose ($n = 20$; $1.98 \times 10^4$ CFU [$1.5 \times 10^4 – 2.69 \times 10^4$]) and were administered after 90-min fasting. Participants were treated with oral antibiotics (ciprofloxacin, 500 mg twice daily, 14 d) at day 14 ($n = 16$; those who did not develop typhoid disease in the first 14 d) after challenge unless diagnosed with acute typhoid infection, in which case treatment commenced immediately ($n = 25$; subsequently termed typhoid diagnosed or TD group). The TD group includes one participant who was withdrawn before day 14 after challenge in the original study (Waddington et al., 2014). Criteria for TD were either microbiological (≥1 positive blood culture collected after day 5) and/or clinical (fever ≥38°C sustained for ≥12 h). Participants were ambulatory and followed up as outpatients at least daily after challenge when safety, clinical, and laboratory measurements were performed (Waddington et al., 2014).

A parallel bicarbonate substudy was performed in which 10 healthy adult volunteers ingested the same volume (120 ml, 17.5 mg/ml) of bicarbonate solution used for neutralization of the stomach acid in the main study. Plasma samples were collected at 0, 12 and 24 h after ingestion, processed and stored at -80°C until further analysis.

Informed consent was obtained from all study volunteers.

**Clinical parameters.** Collection of clinical data including temperature, blood pressure, and heart-rate, and elicitation of symptoms was performed at each participant visit. C-reactive protein (CRP) concentrations were measured at the Oxford University Hospital NHS Trust laboratories by standard procedures, whereas daily blood cultures were performed as previously described and in accordance with national standard operating procedures (England, 2011, 2013; Waddington et al., 2014).

**Gene expression arrays sample processing.** Peripheral venous blood (3 ml) was collected in Tempus Blood RNA tubes (Applied Biosystems) before challenge (baseline, prechallenge controls, D0; $n = 40$), 24 h before TD (TD-24, $n = 23$; TD group only), at TD ($n = 25$; median day of diagnosis = day 7; median day of symptoms = day 7), at 24 h after TD (TD+24, $n = 22$), and 14 d after challenge (D14, $n = 19$). In those who did not develop typhoid fever, gene expression was measured

at day 7 (nD7; $n = 14$) and day 14 (nD14; $n = 16$) after challenge. Total RNA was extracted from all samples using the Tempus Spin RNA Isolation kit (Life Technologies). 50 ng of RNA was used for hybridization into Illumina HT-12v4 bead-arrays (Illumina) at the Wellcome Trust Sanger Institute (Hinxton, England, UK) and fluorescent probe intensities were captured with GenomeStudio software (Illumina).

**Data processing and identification of differentially expressed genes.** Gene transcription responses to infection with wild-type *S.* Typhi were assessed by analysis of changes in transcript abundance in whole-blood samples collected during the study. For each time-point, blood transcriptome profiles were generated by comparing levels of expression to the median of the respective baseline control group. All samples were quantile normalized and background subtracted using the Bioconductor suite in R (Version 3.0.1; Gentleman et al., 2004). For gene level analysis, probes denoted as low quality (illuminaHumanv4.db) were removed and batch correction was applied where necessary using the Combat algorithm (Leek et al., 2012). Data were filtered for detection (P value $\leq 0.05$ in $\geq 60\%$ of samples), and filtered probes were then used for subsequent gene level analysis (*t* test corrected for multiple testing using BH, $P < 0.05$; absolute fold-change (FC), $\geq 1.5$). Data have been deposited at ArrayExpress under accession no. E-MTAB-3423.

**Modular level analysis.** Modular analysis is a systems-scale strategy for microarray analysis that has identified transcriptional modules formed by genes coordinately expressed across multiple disease datasets, thus allowing functional interpretation of the microarray data into biologically useful information. A detailed account of how modules were derived and the module-based mining analysis strategy has been reported elsewhere (Banchereau et al., 2012; Chaussabel and Baldwin, 2014). Modules were deemed activated or suppressed if $\geq 10\%$ genes per module were significantly ($P < 0.05$) over- (red) or underexpressed (blue) compared with prechallenge control samples (Chaussabel et al., 2008). Mean module expression was used as a measure of activation and determined by calculating the mean modular FC expression per sample over controls at a given time points. Percentage expression of each module was identified as the number of over- or underexpressed transcripts divided by the total number of genes contained in a given module.

**Gene expression data typhoid patients in Vietnam.** We used publically available data from a study conducted in Vietnam in 2011 (available from Gene Expression Omnibus under accession no. GSE7000; Thompson et al., 2009). The data used were all healthy control ($n = 16$) samples, as well as samples from acute, culture-confirmed typhoid patients 1 d (D1; $n = 38$) or 3 d (D3; $n = 25$) after admission to hospital (Thompson et al., 2009). We illustrated the overlap between statistically differentially expressed genes in the Vietnam and the challenge study dataset (BH, $P < 0.05$; FC, $\pm 1.25$) using a Venn diagram (Fig. S1 A). To compare the data statistically, we performed GSEA in R using three custom gene sets derived from the Vietnam data. These consisted of all differentially expressed genes, only down- or up-regulated genes (BH, $P < 0.05$; FC, $\pm 1.25$).

**Heat maps and MDTH scores.** As a measure of transcriptional perturbation, weighted MDTH scores (Pankla et al., 2009) were calculated for each sample based on the relevant signatures used by performing outlier analyses on a gene-by-gene basis. Dispersion of baseline control expression values was used to determine whether the values of a given sample and time point (e.g., TD) lay inside or outside the baseline control mean $\pm$ 2SD (Pankla et al., 2009). The heat map in Fig. 1 A was constructed based on genes significantly differentially expressed (BH, $P < 0.05$; FC, $\pm 1.5$) at TD and day 7 (nD7) after challenge in those who stayed well throughout the challenge period, yielding 2,128 genes after duplicates were removed (Euclidean distance, ward clustering).

**Pathway overrepresentation analysis and custom interactome analysis.** Pathway and transcription factor overrepresentation analysis was performed on up-regulated (BH, $P < 0.01$; FC, $>1.5$) and differentially expressed genes (BH, $P < 0.01$; FC, $\pm 1.5$), respectively, at given time points using the publically available overrepresentation tool InnateDB (Lynn et al., 2010). Overrepresentation analysis of the Vietnam dataset was performed using genes up-regulated at days 1 and 3 (BH, $P < 0.01$; FC, $>1.5$). The InnateDB analysis algorithm searches several databases including KEGG, Reactome, Nethpath, PID NCI, Biocarta, and INOH.

A custom network was built by merging the IFN-signaling pathway (Reactome ID: 13077), and the experimentally established interactions of *IDO1* and *AhR* as curated within InnateDB (Lynn et al., 2010) using Cytoscape (Shannon et al., 2003). The interactome was then annotated with transcriptional data from the human challenge model; nodes that were not expressed at all were removed. A gene list was constructed representing genes belonging to the custom interactome, broader annotation of the IFN signaling (Reactome ID: 13077, 18059, and 13074) and the tryptophan metabolism (KEGG:00380). Individual fold-change (FC) gene expression (all challenged volunteers; Fig. 7 C) and protein expression values of macrophage stimulations (two independent experiments) of all genes in this list that were present in the proteomics data were used to perform a principle component analysis (Fig. 7 C, inset).

**Quantitative PCR.** Quantitative PCR was performed on target genes of interest in a subset of samples at prechallenge baselines, TD, and day 7 after challenge in those who did not develop typhoid fever during the 14-d challenge period, and animal tissue or macrophage RNA as indicated. 30 ng RNA was assayed using the TaqMan RNA-to-Ct 1-Step kit (Life Technologies) using TaqMan Gene Expression Assays for tar-

get genes (Life Technologies), following the manufacturer's instructions. Fold-change values over prechallenge baselines were calculated using the $^{\Delta\Delta}$Ct method using GAPDH as a housekeeping gene (Table S8).

**Cytokine assays.** Plasma was separated from 3 ml peripheral venous blood collected in heparinized tubes, treated with proteinase inhibitor, and stored at −80°C. Cytokine concentrations were measured in 50 µl plasma using a custom 18-plex Luminex panel (Table S2; MILLIPLEX MAP kit; Millipore) according to the manufacturer's instructions. Each sample was measured in duplicate and samples with a %CV value >30% were excluded, unless the concentration was between 3.2 and 6.4 pg/ml (two times lower limit of detection [LLD]), in which case 40% was accepted. Cytokine concentrations below the analytes specificity range were set to 50% of that range, unless the value was below the LLD, in which case the concentration was set to 1.6 pg/ml (50% LLD).

**Induced pluripotent stem (iPS) cell–derived human macrophages.** Undifferentiated human iPS cells were maintained on a monolayer of mitotically inactivated mouse embryonic feeder (MEF) cells in advanced Dulbecco's modified Eagles/F12 medium (DMEM/F12) supplemented with 20% knockout replacement serum (KSR), L-Glutamine (2 mM), β-mercaptoethanol (0.055 mM), and 8 ng/ml recombinant human FGF2 (R&D Systems) and differentiated as prescribed previously (van Wilgenburg et al., 2013). Fully differentiated macrophages were primed overnight with 20 ng/ml IFN-γ. On the day of infection, cells were washed with PBS and *S.* Typhi BRD948 (Ty2 Δ*aroA*, Δ*aroC*, Δ*htrA*) or *S.* Typhimurium SL1344 (MOI = 10:1) or IFN-γ (20 ng/ml) was added and incubated at 37°C for 1 h. Unprimed cells were also stimulated using *S.* Typhi BRD948 or *S.* Typhimurium SL1344 (MOI = 10:1) for 1 h. Cells were washed three times and incubated with media containing 50 µg/ml gentamicin to remove extracellular bacteria. After 1 h cells were washed again and fresh medium added. After 4 h, cells were harvested and snap frozen for proteomics analysis, or lysed for RNA extraction or bacterial plating. For inhibitor experiments, IDO1 inhibitor INCB024360 was added at 250 nM final concentration 16 h before stimulation.

**Animal experiments.** WT C57BL/6N mice were infected with $10^4$ CFU *S.* Typhimurium SL1344 grown to log-phase, whereas naive controls received 200 µl PBS ($n$ = 5) by oral gavage. Mice were culled at day 3 ($n$ = 4) and 7 ($n$ = 6) after infection, and cecum, liver, and spleen harvested for RNA extraction and gene expression analysis by qPCR.

Ifngr1$^{tm1Agt}$ mice were obtained from The Jackson Laboratory and backcrossed 10 times onto a C57BL/6J background (hereafter referred to as IFN-γR KO). Six IFN-γR KO mice and six WT mice (in-house) were infected with $10^4$ CFU *S.* Typhimurium SL1344 and monitored daily. Animals were culled when 12% weight loss was observed and cecum, spleen, and liver harvested for RNA extraction. Mice were bred and maintained in accordance with UK Home Office regulations under the project license 80/2596. This license was reviewed by The Wellcome Trust Sanger Institute Animal Welfare and Ethical Review Body (AWERB). Mice were matched for age and sex in each experiment.

**Human iPS-derived macrophage proteomics.** Two biological replicates of iPS-derived macrophages from unstimulated and stimulated conditions (IFN-γ, *Salmonella typhimurium* [STm], and IFN-γ+STm) were lysed and equal amounts of protein extracts were reduced, alkylated and digested with trypsin. The resultant peptides were labeled with the TMT reagents (Thermo Fisher Scientific) according to a modified manufacturer's protocol. The peptide mixture was fractionated with basic reverse phase chromatography over a 35-min gradient program. The liquid chromatography mass spectrometry analysis of the individual fractions was performed on the Dionex Ultimate 3000 UHPLC system coupled with the LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) with a top5 collision induced dissociation higher-energy collisional dissociation sequential activation method. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al., 2014) via the PRIDE partner repository with the dataset identifier PXD001953.

**Human iPS-derived macrophage data analysis.** The acquired mass spectra were submitted to SequestHT search engine implemented on the Proteome Discoverer 1.4 software for protein identification and quantification against a UniProt fasta file containing 20,190 Human reviewed entries. Peptide false discovery rate was set at 0.01 and validation was based on q-value and decoy database search. The analysis resulted in the identification of 20,784 unique peptides attributed to 4,399 unique protein groups of which 4,071 were fully quantified for all the conditions. Protein ratios against the untreated sample were $\log_2$-transformed and median values for the two biological replicates were calculated for each protein. A total of 118, 103, and 136 proteins were found up-regulated >2SD of all protein medians and 58, 55, and 36 proteins were found down-regulated <2SD of all protein medians for the IFN-γ, *Stm*, and IFN-γ+*Stm* treatments, respectively.

**Antibody responses.** Serological data were measured as described by Waddington et al. (2014). In brief, antibody titers in serum against flagellin (H-d–antigen) and lipopolysaccharide (O–antigen) were measured by ELISA at baseline and day 28 after challenge.

**Metabolite analysis in serum.** Kynurenine, quinolate, and tryptophan levels were determined in serum of a subset of participants (TD, $n$ = 8; nTD, $n$ = 7). The metabolites were determined by LC/MS performed by Metabolon Inc. FCs

were generated from median scaled Area Under the Curve (AUC) values (missing values imputed).

**Statistical analysis.** For analysis of the cytokine induction at 12 h after challenge, 12 h cytokine concentrations (pg/ml) were compared with baseline values (unpaired Student's *t* test; Table S4). Linear regression models were fitted to determine the relationship between all cytokines and dose adjusting for baseline (Table S5). Binary logistic regression and Cox proportional hazard models were fitted for all cytokines to determine whether cytokine induction at 12 h predicted subsequent TD or time to diagnosis (Tables S6 and S7). In the proportional hazards model participants without the event (TD) were censored at day 14 after challenge. For analysis of metabolite changes, a paired Student's *t* test (two-sided) was used to determine significant changes from baseline and time point of interest. FCs of kynurenine (TD or nD7 over D0) and quiniolate (TD or nD7 over D0) were correlated with IFN-γ (TD-24 h or nD7 over D0), duration of bacteremia (hours), and maximum temperature change from baseline ($\Delta Temperature_{max}$). All other statistical tests used are stated in figure legends and the text. Statistical analysis was performed using GraphPad Prism v6 or R (Version 3.0.1).

**Online supplemental material.** Fig. S1 compares acute typhoid fever responses in the challenge model with patients from the field. Fig. S2 shows the cytokine responses after challenge. Fig. S3 shows the qPCR validation of array data. Table S1 indicates differential expressed genes at specific time points. Table S2 shows the time points cytokines were measured. Table S3 provides a summary statistic of cytokine data. Table S4 shows p-values for cytokines at 12 h after challenge. Tables S5–S7 shows statistical modeling between cytokine response at 12 h after challenge and several outcome variables. Table S8 indicates primers used in qPCR experiments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20151025/DC1.

## ACKNOWLEDGMENTS

The authors acknowledge the support of the University of Maryland for providing the challenge strain, and the US Centers for Disease Control and Prevention for arranging strain banking and transfer to the UK. Manufacturing of the strain to GMP standard was performed by the Public Health England, Porton Down, and funded by Emergent BioSolutions; we additionally acknowledge the contribution of Dr. Gary Whale in the manufacturing process and development of the release protocols. The authors also acknowledge the kind support of Professor Derrick Crook and the work performed by the Microbiology, Hematology, and Clinical Chemistry laboratories at the Oxford University Hospitals NHS Foundation Trust; Dr. Noel McCarthy and the Thames Valley Health Protection Unit; Professor Vincenzo Cerundolo at the Weatherall Institute of Molecular Medicine for providing access to the Luminex machine, and the willing study participants, their families, and close contacts. The Oxford Vaccine Group acknowledges the support of the National Institute for Health Research Clinical Research Network.

This work was supported by a Wellcome Trust Strategic Translational Award (grant number 092661), the Newton Fund, and the Medical Research Council (MR/M02637X/1). C.J. Blohmke is a Marie Curie Fellow supported by the FP7 program of the European Union. Additional funding was received from the European Molecular Biology Organization (STF to C.J. Blohmke), the National Institute for Health Research Oxford Biomedical Research Centre (Clinical Research Fellowships to C.S. Waddington and T.C. Darton), the Jenner Institute, the Oxford Martin School, the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant numbers R01 AI-036525, U-19 AI-082655, and U19-AI109776 to M.B. Sztein; A. Mejias and O. Ramilo were supported in part by U-19 AI089987, U-19 AI057234, and U01 AI82210). The involvement of D.J. Lynn in this work was supported by European Molecular Biology Laboratory Australia.

The authors declare no competing financial interests.

**Submitted: 22 June 2015**
**Accepted: 8 April 2016**

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