The Hd, Hj, and Hz66 flagella variants of Salmonella enterica serovar Typhi modify host responses and cellular interactions

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Salmonella Typhi, the causative agent of typhoid fever, is a monophasic, human-restricted bacterium that exhibits limited phenotypic variation. S. Typhi from Indonesia are a notable exception, with circulating strains expressing diverse flagella antigens including Hj, Hd and Hz66. Hypothesizing that S. Typhi flagella plays a key role during infection, we constructed an S. Typhi flIC mutant and otherwise isogenic S. Typhi strains expressing the Hj, Hd, Hz66 flagella antigens. Phenotyping revealed differences in flagellum structure, strain motility and immunogenicity, but not in the ability of flagellated isolates to induce TLR5 activity. Invasion assays using epithelial and macrophage cell lines revealed differences in the ability of these S. Typhi derivatives to invade cells or induce cellular restructuring in the form of ruffles. Notably, the Hj variant induced substantial ruffles that were not fully dependent on the GTPases that contribute to this process. These data highlight important differences in the phenotypic properties of S. Typhi flagella variation and how they impact on the pathogenesis of S. Typhi.

Typhoid fever, the disease caused by the bacterium Salmonella enterica serovar Typhi (S. Typhi), remains common in locations with poor sanitation. The clinical syndrome of typhoid, with the characteristically high fever, is induced by the infecting bacteria invading the gastrointestinal surface and spreading systemically in the bloodstream. The disease is seldom fatal if treated with appropriate antimicrobials but can become life-threatening, with some patients developing complications, such as intestinal perforation and neurological symptoms.

S. Typhi is routinely identified and classified by the Kauffman-White scheme using specific typing sera. The major typing antigens for S. Typhi are the O9 and O12 (epitopes present on the O antigen side-chain of LPS), the Vi or virulence-associated polysaccharide capsule, and the flagella (H) antigen, predominantly of type Hd. S. Typhi is a monophasic, human-restricted pathogen, and all extant organisms originate from a single common ancestor that crossed into the human population thousands of years ago. Consequently, the genomes of individual S. Typhi are highly conserved, exhibiting limited evidence of recombination, isolate specific horizontal gene transfer, or geographically restricted pathovars.

Flagellin is the monomer of the flagella filament, the dominant protein of a complex super-molecular structure, the flagellum, which is essential for bacterial motility and chemotaxis. Flagellin is a key trigger of the immune response since this polypeptide engages both the innate, through Toll-like receptor 5 (TLR5), and the adaptive arm of the mammalian immune system. The majority of Salmonella serovars are biphasic, and in a process called phase variation exhibit the ability to switch expression between two alternative flagellin genes, flIC and fljB, which encode the phase 1 and 2 flagella. Salmonella flagella phase variation is controlled by the invertible promotor (hin), which influences the pattern of flIC and fljB transcription. FljA is a repressor of flIC transcription, ensuring that only a single flagellin gene is expressed at one time. S. Typhi is atypical with respect to most Salmonella in that it generally possesses the flIC Hd encoding flagellin gene only. However, S. Typhi variants...
originating in Indonesia harbor pBSSB1, a linear plasmid encoding a
*fljB* analogue that directs the expression of the *Hz66* flagellin anti-
gen12,13. Plasmid pBSSB1 additionally encodes a repressor of the
chromosomal *fljC* gene; ensuring only one flagella antigen is
expressed at a time. A third antigenic variant of flagellin, known as
*Hd*, is also found in some *S. Typhi* that also originate from Indonesia.
*Hj* is encoded by an allele of *fljC* gene harboring a 261 bp in-frame
deletion in the central region of the *Hz66* *fljC* coding sequence14.

The impact of flagella antigenic variation on pathogenesis and
immunity within *S. Typhi* is not well described. Furthermore, we
hypothesized that flagella variation plays a unique role in regulating
the immune response to *S. Typhi* infection in Indonesia, a location
where a range of atypical *S. Typhi* flagella variants circulate.
Here, we have engineered an aflagellated *S. Typhi* Δ*fliC* mutant and a set of
three otherwise isogenic derivatives of *S. Typhi* that differ only in the
flagella antigen variant expressed on the surface (*Hd*, *Hj* and *Hz66*).
These isogenic *S. Typhi* derivatives were subjected to a range of
phenotypic assays including their ability to interact with epithelial
cells and macrophages. We show that the flagella type can influence
the immune response during typhoid and impact on the ability of *S.
Typhi* to invade host cells.

**Results**

**Construction and primary characterization of isogenic *S. Typhi*
flagella variants.** To compare the properties of the *Hd*, *Hj* and *Hz66*
flagella, we used targeted mutagenesis to construct three otherwise
isogenic *S. Typhi* derivatives that differed only in terms of their
flagellin gene content (alignments of the flagellin proteins from *S.
Typhi* and *S. Typhimurium* are shown in Figure 1). For consistency,
all gene replacements targeted the *fliC* locus, i.e. the *Hd* encoding *fljC
*gene was completely replaced with only the *Hj* *fljC* or the *Hz66* *fljB
*allele using the native *fliC* promoter to direct expression. An
additional isogenic derivative harboring a null deletion in *fliC
(Δ*fliC*) was constructed to serve as an amotile, aflagellated control.
The genetic structure of these flagellated and aflagellated *S. Typhi*
derivatives were confirmed by sequencing and the different derivatives
were then screened in agglutination assays with flagella specific antisera and were found to express the appropriate flagella
antigens (Figure 2).

All three *S. Typhi* derivatives harboring functional flagellin genes
expressed peritrichous flagella observable by negative staining under
the transmission electron microscope (TEM) (Figure 2a), while the
Δ*fliC* had no detectable flagella. *S. Typhi* *Hd* and *Hj* expressing
derivatives elaborated nine (range: *Hd*; 3–18, *Hj*; 5–16) and the
*Hz66* variant a median of seven flagella per cell (range; 4–14)
(Figure 2b). The *Hz66* flagella had a significantly greater diameter
(median: 13.1 nm, range: 12.1–14.9 nm) than both the *Hd* and *Hj
flagella (median: 12.1 nm, range: 10.6–13.7 nm, and median;
8.74 nm, range: 8.19–9.77 nm respectively) (*p*<0.0001; 2 sided t-
test) (Figure 2c and 2d). Furthermore, the *Hj* flagella were signifi-
cantly shorter in length (median; 2.06 μm, range; 0.33–8.16 μm)
than both the *Hd* (median: 4.05 μm, range 0.45–10.2 μm) and
*Hz66* flagella (median; 4.65 μm, range; 0.545–10.3 μm) (*p*<0.0001;
2 sided t-test). The difference in length between the *Hd* and the *Hz66
flagella was not significant.

The motility of the *S. Typhi* flagella variants was measured by
assessing their swimming capabilities in soft media over a defined
incubation period. The flagellated *S. Typhi* Δ*fliC* derivative was amot-
tile, while each of the flagellated *S. Typhi* swam between 37 and
58 mm in the agar matrix during the 16-hour incubation period at
37°C (Figure 2e). The *Hz66*-expressing *S. Typhi* derivative was con-
sistently the least motile. Despite having the shortest flagella, the *Hj* *S.
Typhi* derivative migrated significantly further in the soft agar (med-
ian; 57 mm, range; 57–58 mm) than the *Hd* and the *Hz66* derivatives
(median; 50 mm, range; 49–51 mm, median; 38 mm, range 37–
41 mm, respectively) (*p*<0.0001; 2 sided t-test).

**Indonesian Typhoid patients elaborate IgG against *Hd*, *Hz66* and
*Hj* flagellin.** Flagellin is highly immunogenic and antibodies (IgG)
against *S. Typhi* flagellin can be measured for a prolonged period
after a confirmed typhoid infection15. As the isogenic *S. Typhi*
derivatives demonstrated different phenotypic qualities we
hypothesized that the organisms expressing these flagella antigens
might stimulate different responses from the immune system during
natural infection. To test this hypothesis, IgG against *Hd*, *Hj*
and *Hz66* flagellin was measured in a group of typhoid patients. Firstly,
the type of flagella genes encoded in *S. Typhi* isolated during a
typhoid case/control study conducted in Jakarta, Indonesia, a
region where *S. Typhi* expressing the *Hd*, *Hj* and *Hz66* flagella are
circulating, were assessed by PCR amplification. Thirty *S. Typhi
isolates, where a corresponding acute serum sample from a typhoid
patient was available, were analyzed and 15 were *Hd*, 4 were
*Hz66*, 11 were *Hj*,Hz66, and none were *Hj* only (Table 1). Available
disease metadata was stratified by flagellin variant and
there was no significant difference between the three groups and
the number of days of fever prior to hospitalization. However,
*Hz66*-positive *S. Typhi* originated from on average older patients
than the isolates expressing *Hd* alone by a mean of seven years
(*p*=0.014; 2 sided t-test).

Serum from the 30 from typhoid patients from which the above *S.
Typhi* flagella variants were isolated and 79 from asymptomatic con-
trols (Table 1) were screened using an ELISA to measure IgG against
the three forms of *S. Typhi* flagellin (Figure 3). The majority of
typhoid patients (25/30), regardless of the *S. Typhi* flagella variant
isolated from their blood, harbored IgG against *Hd* (Figure 3a).
Furthermore, the preponderance of patients (12/15) infected with
an *Hz66* *S. Typhi* harbored IgG against *Hz66*, as did four patients
infected with *Hd* isolates (Figure 3c). Conversely, only three patients
demonstrated an anti-*Hj* IgG response, of which only one was
infected with an *S. Typhi* *Hz66*/*Hj* isolate (Figure 3b). Interestingly,
the asymptomatic controls also harbored antibody responses of a
similar magnitude to the typhoid fever patients, indicating probable
previous exposure. Of the 79 asymptomatic controls, 31 (39.2%) harbored
IgG against at least one of the flagellin antigens, with the
majority responding to *Hd* (30/31, 97%), four (5.1%) to *Hz66* IgG
with only one of these to *Hz66* exclusively and also to *Hd*. Only one
control sample harbored measurable anti-*Hj* IgG and this also harb-
ored IgG against the other two flagellin antigens. We found no significant
difference in IgG levels to *Hd*, *Hj* or *Hz66* flagellin between the
asymptomatic controls or those infected with *Hd*, *Hj*
or *Hz66* *S. Typhi* (*p*>0.05 in all pairwise comparisons; 2 sided t-
tests).

**S. Typhi flagella variants induce similar activation of TLR5.**
Predicting that the *S. Typhi* flagella variants may have a differing
ability to stimulate Toll Like Receptor 5 (TLR5), we independently
c-transfected HEK293 cells expressing TLR5 with the three *S.
Typhi* flagella variants, *S. Typhi* Δ*fliC* or *S. Typhimurium SL1344 as a
positive control (Figure 4). All of the *S. Typhi* derivatives, with
notable exception of the Δ*fliC* mutant, were able to stimulate TLR5
and produce downstream NFκB signaling with a similar degree of
potency.

**The interaction of *S. Typhi* flagella derivatives with macrophages
and epithelial cells.** The *S. Typhi* derivatives were inoculated onto
THP-1 cells to measure cellular uptake and *S. Typhi* expressing the
*Hd* flagella were consistently taken up by more efficiently than the
*Hj* and *Hz66* expressing derivatives (Figure 5a). The Δ*fliC* *S. Typhi* was
reproductively taken up at a lower frequency than any of the flagellated
derivatives. No difference in cytotoxicity was observed between the *S.
Typhi* derivatives using a lactate dehydrogenase assay (data not
shown).

The transcriptome of THP-1 cells infected with the *S. Typhi
derivatives was measured by DNA microarray analysis of the host mRNA
populations. S. Typhi expressing Hf and Hj flagellin induced highly similar transcription patterns in host cells, with higher numbers of differentially expressed genes, compared to the Hz66 or DfliC derivatives (Table 2). The pathway and gene ontologies of the differential expressed genes groups were determined using InnateDB and could be divided into three main groups with corresponding profiles (Figure 5b). These three main groups were; i) the up-regulation of genes involved in inflammation in cells infected with flagellated bac-

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**Figure 1 | Amino acid sequence alignment of S. Typhi and S. Typhimurium flagellin proteins.** Amino acid alignment of the flagellin genes (from top to bottom) FlcC Hz66 (S. Typhi), FljB (S. Typhimurium), FlcC (S. Typhimurium), FlcC Hf (S. Typhi) and FlcC Hj (S. Typhi).
teria; ii) the up-regulation of gene involved in gene expression, translation, and protein metabolism in cells infected with either Hd or Hj derivatives and; iii) the proportional down-regulation of genes in the NOTCH pathway, Wnt-mediated gene transcription, and protein kinase activity with flagellated organisms.

Next, the various flagella variants were independently inoculated onto human epithelial-like Hep-2 cells (Figure 6). The Hd and Hj derivatives invade Hep-2 cells with a comparable frequency but the Hz66 derivative reproducibly demonstrated a significantly higher capability for invasion (0.42% ± 0.15) (Figure 6a).

Table 1 | The characteristics of control subjects and typhoid fever cases infected with S. Typhi expressing combinations of the three flagella variants

| Group                      | N  | Male sex (%) | Median age in years (range) | Median days of fever (range) |
|----------------------------|----|--------------|-----------------------------|-------------------------------|
| Typhoid with S. Typhi Hd   | 15 | 8 (53)       | 19 (10–35)                  | 5 (3–14)                     |
| Typhoid with S. Typhi Hd:Hz66 | 4  | 2 (50)       | 23.5 (15–30)                | 5 (5)                        |
| Typhoid with S. Typhi Hj:Hz66 | 11 | 7 (64)       | 29 (11–57)                  | 4 (3–30)                     |
| Community controls         | 79 | 23 (29)      | 24 (7–74)                   | NA                           |
Figure 3 | The antibody response to Hd, Hj, and Hz66 flagellin in Indonesian subjects. Anti-flagellin IgG antibody titers in serum from typhoid fever patients (n=30; 15 Hd, 4 Hj Hz66, 11 Hd Hz66) and community controls (n=79) in Indonesia. a) Scatterplot of IgG measurements against Hd flagellin in patients infected with Hd, Hj Hz66, or Hd Hz66 S. Typhi and controls. b) Scatterplot of IgG measurements against Hj flagellin in patients infected with Hd, Hj Hz66, or Hd Hz66 S. Typhi and controls. c) Scatterplot of IgG measurements against Hz66 flagellin in patients infected with Hd, Hj Hz66, or Hd Hz66 S. Typhi and community controls. Titers measured as the log10 of the highest dilution with an OD three times the OD value of negative controls.

Furthermore, S. Typhi ΔfliC was reproducibly even less invasive and was only marginally more invasive than S. Typhi ΔinvA. We found no significant difference in the ability of the various flagellated derivatives or ΔfliC and ΔinvA S. Typhi to attach to the epithelial cells (Figure 6b).

Similar to the work on THP-1 cells, gene expression profiles were determined using microarray analysis of mRNA populations present in epithelial cells exposed to S. Typhi expressing the different flagellin. Levels of IL8 mRNA and pathways related to cytokine-cytokine receptor interactions and the HIF1α transcription factor network were comparatively over expressed by Hep-2 cells exposed to flagellated S. Typhi compared to the ΔfliC derivative. Unsupervised hierarchical clustering revealed a similar response to S. Typhi expressing either Hd or Hj but this was distinct from S. Typhi Hz66 (Figure 6c). In fact, the overall host transcriptome response to the Hz66 was more comparable to the response induced by S. Typhi ΔfliC and ΔinvA than the Hd and Hj derivatives (Table 2). This difference was mainly restricted to the up-regulation of genes involved in gene expression, translation and general protein metabolism e.g. genes CDK1, CDKN1B, MNAT1, CCNG1, CCNG2, RPL7, RPL9, RPL14 and MYC (Supplementary information).

During epithelial cell invasion, Salmonella trigger rearrangements of the host cell cytoskeleton including ruffles in the plasma membrane. SEM was used to compare the interaction of S. Typhi expressing Hd, Hj or Hz66 flagellin with epithelial cells during invasion and to gain insight into the different gene expression profiles. All S. Typhi were able to stimulate substantial membrane ruffling but abnormally large ruffles were consistently observed when S. Typhi expressing Hj flagella interacted with epithelial cells (Figure 7a).

Ruffles are triggered in part by effectors secreted through the Salmonella Pathogenicity Island 1 (SPI1) interacting with host Rho-GTPases, including Rac1, Cdc42, and RhoG16–18. To assess the contribution of each of these Rho-GTPases to ruffling, siRNAs were generated for each gene and ruffling was observed on Hep-2 cells exposed to individual siRNAs using TEM. siRNAs to RAC1, CDC42 or RHOG completely repressed ruffle formation on Hep-2 cells exposed to individual siRNAs using TEM. siRNAs to RAC1, CDC42 or RHOG completely repressed ruffle formation on Hep-2 cells exposed to S. Typhi expressing the Hd or Hz66 flagella (Figure 7b). Cell ruffling was reduced when Hj S. Typhi were inoculated onto Hep-2 exposed to RAC1 but interestingly not RHOG and CDC42 siRNAs.

Discussion

We performed a series of experiments using a combination of typhoid patients and in vitro assays to assess the impact of flagella type on host cell-pathogen interactions involving S. Typhi. To facilitate these studies we constructed a novel series of carefully engineered isogenic S. Typhi derivatives differing only in the antigenic structure of their flagella. These data are of interest as the majority of global S. Typhi isolates are monophasic and express only the classical Hd flagella yet novel S. Typhi are originating in the Indonesian archipelago that can express alternative flagellin either from an allelic variant of Hd invA or from a novel fliB gene encoded on a linear plasmid pBSB1, known as Hz6613–14. Here we show that the different flagellin have distinct structural features that directly impinge on the motility of the bacteria and their pathogenic potential. Firstly, we found that Hz66 flagella were measurably thicker than Hj or Hd flagella and that this property translated into poorer motility when comparing S. Typhi ΔfliC and ΔinvA derivatives or Hj S. Typhi Hz66 and ΔfliC to induce TLR5 activity after bacterial inoculation on to transfected HEK293 cells compared to an S. Typhimurium positive control. Results are measured as NFκB-luciferase activity relative to Renilla-luciferase activity per 10⁶ cfu mL⁻¹ of infecting bacteria.
Figure 5 | The interaction of S. Typhi flagella derivatives with macrophages. The monocytic THP-1 cell line was differentiated into macrophages and infected with S. Typhi expressing one of the three-flagellin variants and the non-flagellated mutant (ΔfliC) (a) Histogram showing the median proportion of recovered (intracellular) bacteria normalized by the inoculum and averaged over six experiments at 1 and 2 hours post infection. Asterisk highlights statistically significant variations by pairwise comparison (p < 0.001), error bars represent one standard deviation. (b) THP-1 cells were infected and RNA was isolated for microarray analysis of host mRNA population. Figure shows hierarchical clustering of differentially expressed genes in THP-1 cells infected with the three flagellin derivatives, and the non-flagellated mutant (ΔfliC), compared with uninfected THP-1 cells. The cut-off for differentially expressed genes was an absolute fold change >2 and FDR corrected p-value of <0.05. Red/green color scale indicates level of gene expression, red indicates increased gene expression, and green indicates reduced gene expression.

Table 2 | The differential gene expression changes induced by S. Typhi flagella derivatives in THP1 and Hep2 cells

| S. Typhi type | THP1 |  |  | Hep2 |  |  |
|--------------|------|---|---|------|---|---|
|              | Up regulated | Down regulated | Total | Up regulated | Down regulated | Total |
| **Hd**       | 200   | 100 | 300 | 537   | 169 | 706 |
| **Hj**       | 228   | 5   | 233 | 480   | 220 | 700 |
| **Hz66**     | 32    | 9   | 41  | 153   | 120 | 273 |
| **ΔfliC**    | 16    | 8   | 24  | 117   | 196 | 313 |
| **ΔinvA**    | NA    | NA  | NA  | 129   | 132 | 261 |

*Table shows the number of differentially expressed genes in infected cells, compared to an uninfected control. The cut-off values for differentially expressed genes is a ≥2 fold change and an FDR corrected p-value of ≤0.05.
Typhi Hz66 to Hd and Hj-positive derivatives. Correspondingly, S. Typhi Hj produced shorter and thinner flagella structures and swam faster than both Hd and Hz66 S. Typhi derivatives. During laboratory observation the Hj flagella were found to be more fragile than the other flagella variants and were detected mainly in the culture medium whereas the other flagella were predominantly attached to the bacterial body. This data are broadly in keeping with previous observations on S. Typhimurium flagellin genes with deletions approximately the same size and location as Hj19. However, our results are somewhat different some previously published observations, in which non-isogenic Hd strains were reported to have a higher motility20 but here isogenic derivatives were used to compare these phenotypic characteristics.

Data collected using Hep-2 or THP-1 cells indicated that the type of expressed S. Typhi flagella can dramatically influence host/pathogen interactions. These results were supported by our unpublished observation that S. Typhi ΔfljK derivatives that produce and secrete unpolymerized flagellin also exhibit a reduced ability to invade Hep-2 cells, in keeping with the impact of flagellin in S. Typhimurium pathogenesis21,22. The increased capacity of the S. Typhi Hj to invade Hep-2 cells appears to correlate with the induction of larger cellular actin ruffles than those induced by either Hd or Hz66 S. Typhi derivatives. The formation of these ruffles is associated with invasion, triggered when Salmonella have intimate contact with a non-phagocytic cell and involves a number of Salmonella Pathogenicity Island associated effector proteins23. These effectors interact with Rho GTPases within the host cell, stimulating a rearrangement of actin in the host cell cytoskeleton16–18. To investigate the mechanism of ruffle formation we performed a number of iRNA experiments to suppress the expression of Rho GTPases, which are known to interact with Salmonella effectors (RHOG, RAC1, CDC42). The resulting data demonstrated that the dramatic ruffle formation induced by S. Typhi Hj occurred independently of CDC42 and RAC1. These data predict the existence of an alternative signaling cascade resulting in bacterial internalization, which is activated through contact with bacterial flagellin. In support of this hypothesis, it has been shown that S. Typhimurium flagellin is injected into the host cell cytosol in part through SPI124, and that flagellin may be acting as an ‘effector protein’ during cellular invasion and uptake.

As with our observations in epithelial cells, the presence of flagella was required for the efficient uptake of S. Typhi into macrophages. Here, we also observed differences in invasion rates between the S.
Typhi flagellated derivatives but here the S. Typhi H\textsubscript{d} derivative had a greater capacity for internalization than the S. Typhi H\textsubscript{j}. This differential interaction between the H\textsubscript{d} and H\textsubscript{j} derivatives with macrophages may, in part, be one of the factors influencing why H\textsubscript{d} S. Typhi are successfully globally whereas H\textsubscript{j} derivatives predominantly restricted to the islands of Indonesia. However, additional factors such as host genetics and environmental conditions may play an even greater role in this atypical geographic restriction. Others have suggested a role for the predatory protozoan species carried by Indonesians\textsuperscript{25}. A role for immune invasion in the evolution of the novel Indonesian S. Typhi remains unproven, although the apparent poor immunogenicity of H\textsubscript{j} may be contributing to local selection on S. Typhi. Here, we speculate that the Hz66 variant is moving into a niche in individuals who have been exposed to H\textsubscript{d} S. Typhi.

We found a wide variation in gene expression patterns between cells exposed to the S. Typhi flagella derivatives. For example, the S. Typhi Hz66 derivative consistently induced transcriptome profile more similar to Δ\textit{fliC} and Δ\textit{invA} than the H\textsubscript{d} and H\textsubscript{j} S. Typhi, which were in turn more similar. All the flagellated bacteria, however, induced a robust and measurable acute inflammatory response, in keeping with the lack of detected difference in signaling through TLR5.

**Figure 7** The ability of S. Typhi flagella derivatives to induce ruffling in epithelial cells. (a) SEM images of Hep-2 cells infected with S. Typhi H\textsubscript{d}, H\textsubscript{j}, and Hz66 flagella derivatives. Images show ruffles induced by S. Typhi inducing cytoskeletal rearrangement, exaggerated by S. Typhi H\textsubscript{j}. (b) The role of Rho GTPases during ruffle formation was assessed by using siRNA to block the RAC1, CDC42, and RHOG Rho GTPases. Images prepared by SEM of Hep-2 cells with knock down Rho GTPases or controls infected with S. Typhi. (c) Western blot of cell lysates, confirming the lack of Rho GTPases expression after siRNA. Lanes, 1; untreated cells, 2; reagent control, 3; non-interfering siRNA control, 4; CDC42 siRNA, 5; RAC1 siRNA and 6; RHOG siRNA.
Indonesia is still endemic for typhoid fever, with an estimated incidence of 810/100,000 cases per year\textsuperscript{26}. The number of individuals within the community with a substantial IgG response to S. Typhi flagellin presumably reflects this high prevalence of typhoid in Indonesia. The serum from several individuals (both typhoid fever patients and community controls) exhibited signatures correlating with multiple infections with S. Typhi expressing different flagellin antigens. This theory of multiple infections is supported by the difference in median ages, since those infected with Hj or Hj66 S. Typhi were significantly (for Hj66) older than those with Hj infections. These data suggest that Hj66 S. Typhi may be more ‘opportunistic’ than S. Typhi Hj, exploiting a niche after a previous infection/exposure to S. Typhi Hj. An additional observation was that both Hj and Hj66 flagellin appears to induce a more robust IgG response than Hj. It is noteworthy that when mice were immunized with Hj flagellin they mounted a much weaker antibody response compared to similar mice immunized with Hj66 or Hj flagellin (our unpublished observations). This reduced immunogenicity of Hj flagellin is perhaps not surprising, since the single dominant B cell epitope of Hj66 is centered at residues 229–230, within in the section missing in Hj (aa 224–310)\textsuperscript{27}.

In summary, the data presented here indicates the important, active role of flagellin in host pathogen interactions during S. Typhi infection, engaging both innate and adaptive branches of the immune response. The differences in invasion and immunogenicity observed between flagellin variants suggests an almost opportunistic behavior of the less widespread variants (Hj and Hj66), taking advantage of preexisting anti-Hj immunity; an issue that should be taken into account when developing novel whole-cell flagellin Salmonella vaccines.

**Experimental procedures**

**Bacterial and genetic manipulation.** The attenuated S. Typhi Ty2 derivative BRD948 (Hlj), harboring deletions in the gyrA, aroC and htrA genes, was used for all experimental studies related to genetically engineered organisms with a containment level three organism\textsuperscript{28}. S. Typhi BRD948 and derivatives are approved for use in a containment level two laboratory in the United Kingdom\textsuperscript{29}. All genetic manipulations were performed using Luria–Bertani (LB) media supplemented with 40 mg L\textsuperscript{-1} of 1-phenylalanine and l-tryptophan, and 10 mg L\textsuperscript{-1} of p-aminobenzoic acid and 2,3-dihydroxybenzoic acid (aroX mix). When required, media was supplemented with chloramphenicol, ampicillin or kanamycin and growth temperatures were adjusted (37°C or 42°C). Treatment of the plasmid was prepared using Gene Modification Kit (Euroclone) or plasmids were electroporated in S. Typhi Typhoid Ty4.1. PCR amplifications were performed using Phusion High-Fidelity (New England BioLabs) or PCR amplification and sequencing of the Hj and Hj66 encoding genes were inserted correctly.

**Cellular invasion assays.** Hep-2 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Sigma), 10% fetal calf serum (FCS, Sigma) and 2% L-glutamine (Sigma), at 37°C in 5% CO\textsubscript{2}. The day before the infection assays were performed, cells were seeded at 1 × 10\textsuperscript{4} cells/well in 24-well plates and incubated overnight. Bacteria were added at a multiplicity of infection (MOI) of 100:1 (bacteriophage). Plates were then centrifuged at 600G for 5 minutes to ensure contact with cells. Cells were incubated for 2 hours, washed with PBS and fresh medium supplemented with 100 µg mL\textsuperscript{-1} gentamicin was added. Cells were further incubated for three hours. After washing, cells were lysed with 0.1% Triton X-100. Serial dilutions were performed and plated on agar plates for enumeration after overnight incubation. For adhesion/invasion assays, Hep-2 cells were incubated at 4°C for 20 minutes prior to infection. Bacteria were resuspended in ice-cold DMEM and added at a MOI of 100:1. Plates were incubated for 1 hour at 4°C to allow attachment but not invasion; cells were then washed and lysed as described above. For the invasion assays, plates were washed and fresh pre-warmed DMEM was added to the wells. Cells were then incubated for one hour at 37°C. The media was then changed to DMEM with gentamicin and cells were incubated for another hour, before washing and cell lysis as described above.

THLz1 cells were cultured in RPMI-1640 medium (Sigma), containing 10% FCS, 2 mM L-Glutamine, at 37°C and in 5% CO\textsubscript{2}. A week prior to infection, THP-1 cells were seeded onto 24-well plates, 1 × 10\textsuperscript{4} cells/well, and differentiated into macrophages with 50 ng mL\textsuperscript{-1} of phorbol 12-myristate 13-acetate (PMA, Sigma). Before adding the bacteria, cells were washed with PBS and fresh RPMI was added. Infections with bacteria were described above. Cells was incubated for 2 hours. The media was then changed to RPMI supplemented with gentamycin. The cells were then incubated further for selected periods of time and then washed and lysed as described for Hep-2 cells.

**Electron microscopy.** For microscopic analysis of infected cells, cells were seeded onto glass coverslips and infections were performed as described above. Samples were prepared for scanning electron microscopy (SEM) as previously described\textsuperscript{30} and for transmission electron microscopy (TEM) negative staining as previously described\textsuperscript{31}. Images were collected using a 120 kV FEI Spirit Biotwin with a Tietz F4.15 CCD camera and the field dimensions calculated using version 3 of TEM Tecnai software.

**Ruffling signaling cascade.** A day prior to transfection, 2 × 10\textsuperscript{5} Hep-2 cells per well were seeded into 24-well plates. Immediately prior to transfection, fresh media was added to the wells. Transfections were performed using Lipofectamine\textsuperscript{TM} RNAiMAX (Invitrogen), as per manufacturer instructions. Cells were transfected with 3 pmol siRNA (Dharmacon, RAC1: pool of D-003560-05, D-003560-07, D-003560-08, D-003560-09; CDC24: pool of D-003557-01, D-003557-02, D-003557-03, D-003557-04; RHOG: pool of D-008995-01, D-008995-02, D-008995-03, D-008995-04; siRNA control: ON-TARGETplus Non-Targeting Pool, D-001810-10) for 48 hours. After successful transfection, cells were infected with S. Typhi as described above for a period of one hour. After infection, cells were washed with PBS and processed for Western blotting and SEM. For Western blotting, cells were lysed with 109 µL lysis buffer (10 mM Tris–Cl pH 7.6, 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, complete mini EDTA–protease inhibitor (Roche)) and then centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was recovered and mixed with loading buffer. SDS-PAGE was performed using 15% acrylamide gels. Membranes were incubated in 5% nonfat dry milk in TBS+0.1% Tween 20 (anti-RAC1 (1/1,000), Millipore); anti-CDC24 (1/1,000, rabbit, Cell Signalling Technologies); anti-RHOG (1/250, rabbit, Santa Cruz Biotechnologies) and anti-actin (1/10,000, rabbit, Sigma) were used as primary antibodies.

**Microarray analysis.** Both THP-1 and Hep-2 cells were infected with and infected with S. Typhi as described above for a period of one hour. The strains used for infection were BRD948, BRD948-Hj, BRD948-Hj66, BRD948-Hj66 and, in the case of Hep-2 cells, BRD948-Ah7A. An uninfected control well was also included. After infection, the cells were washed and RNA was purified using the RNeasy Mini Kit (Qiagen), as per manufacturer’s instructions. RNA samples were then amplified and labeled using the TotalPrep 96 kit (Ambion, Austin, TX, USA) and hybridized onto Illumina\textsuperscript{TM} Human HT-12, V4 Beadchips (Illumina, San Diego, CA, USA). The chips were scanned on an Illumina BeadArray Reader and raw intensities were extracted using Illumina BeadStudio Gene Expression Module. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2395.

Normalization and data analysis of the microarrays were performed using GeneSpring X software (Agilent Technologies). A quantile normalization using a baseline correction from the median of all samples was performed. For each comparison, differentially expressed genes were defined as those exhibiting a fold change ≥ 2 and a FDR (false discovery rate) corrected p-value ≤ 0.05. Adjusted p-values were calculated using the Benjamini and Hochberg method\textsuperscript{32}. Pathway, gene ontology (GO), and interaction analysis was performed using InnateDB (www.innatedb.ca). Over-represented pathways or GO terms were deemed significant if having a FDR corrected p-value ≤ 0.05.

**TLR5 signaling.** TLR5 signaling was measured by using HEK293 cells (human embryonic kidney cells, ATCC number CRL-1573) transfected with a TLR5 expression vector, an NF-kB-luciferase reporter (firefly luciferase, Stratagene) a Renilla-luciferase reporter as a transfection control (Promega), and a ‘filler’ plasmid pEF-BOs, using Fugene 6 (Roche). Twenty-four hours after transfection, the cells were infected with serially diluted heat-killed culture of the relevant bacteria (BRD948-Hj, BRD948-Hj66, BRD948-Ah7A). Luciferase activity was measured using the Luciferase Assay System (Promega) as per manufacturer’s instructions. The optimal bacterial dilution was defined as the dilution at which the greatest differential in the expression of the two luciferases for the positive control was provided. The same bacterial concentrations for the different mutants were then compared for pFNkβ-luciferase expression; results are presented as NF-kB-luciferase activity relative to Renilla-luciferase activity.
serology are as previously described26,31,35. Briefly, the serum from the typhoid cases
were added by PCR amplification to detect the nature of the native flagellin
gene(s). For this work, thirty typhoid cases with known flagella variants and 79
randomly chosen community controls were selected for serological analysis.
For the ELISA assays, Nunc MaxiSorp (Thermo Scientific) or Microlon (Greiner)
96-well plates were coated with 2 µg ml−1 of S. Typhi flagella antigens (Hd, H) or
Hs66, purified as described previously in phosphate buffer pH 9.5 and incubated
overnight at 4 °C. The plates were blocked with 1% BSA (Sigma) in PBS-0.05% Tween
(Sigma) for 1 hour at 37 °C. Sera were added in serial dilutions in PBS-0.05% Tween-
0.1% BSA and incubated for two hours at 37 °C. The secondary antibodies (rabbit
anti-human IgG-HRP conjugated (Oako), mouse anti-human IgG-biotin conjugated
(Sigma) or mouse anti-human IgG-biotin conjugated (Sigma)) were added at a
1/1,000 dilution in PBS-0.05% Tween-0.1% BSA and incubated for two hours at 37 °C.
Plates were developed with OPD (SIGMAFAST™ OPD, Sigma) at room temperature
for 10 minutes, following the manufacturers’ instructions. The reaction was stopped
with 20 µl/well 3 M H2SO4. Plates were read at 490 nm using an ELISA plate reader.

Statistical analysis. Statistical tests were applied to determine differences between
the strains with respect to flagella morphology, motility, and cellular invasion. The
nature of these tests is outlined in the results with the corresponding
p-values. ANOVA and 2-
sided t-tests were performed using GraphPad Prism (GraphPad Software Inc.),
for multiple of pairwise comparisons, respectively. No correction for multiple testing was
applied. p values of ≤ 0.05 were considered to be statistically significant.

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