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Hemoglobin Transforms Anti-Inflammatory Salmonella typhi Virulence Polysaccharide into a TLR-2 Agonist

Rohini Garg and Ayub Qadri

Vi capsular polysaccharide is a major virulence determinant of the human typhoid-causing pathogen Salmonella typhi; it is absent in nontyphoidal Salmonella serovars. We show in this study that through its specific interaction with the membrane recognition complex containing the prohibitin family of molecules, Vi can inhibit the production of inflammatory cytokines from mononuclear phagocytes stimulated with Salmonella flagellin. Remarkably, Vi lost this anti-inflammatory capability and switched to a proinflammatory state when cell stimulations were performed in the presence of serum. The serum-transformed proinflammatory form of Vi induced secretion of cytokines from monocytes by specifically engaging TLR-2/TLR-1. The molecule responsible for bringing about this conversion of Vi from an anti-inflammatory to a proinflammatory form was serum-derived hemoglobin. Derivatives of Vi incapable of interacting with hemoglobin did not switch to a proinflammatory state in vitro or in vivo. These findings provide compelling evidence for a role of hemoglobin in transforming the anti-inflammatory S. typhi virulence polysaccharide into an immune activator. The Journal of Immunology, 2010, 184: 000–000.

Microbial pathogens are sensed by the host immune system through germline encoded pattern recognition receptors (PRRs) including TLRs, nucleotide binding and oligomerization domain-like receptors (NLRs) and lectins (1). These receptors recognize conserved pathogen-associated molecular patterns that include lipids, polysaccharides, proteins, and nucleic acids (1). In addition to these cell membrane-associated and cytosolic sensors, several circulating host factors have been shown to enhance inflammatory responses produced by microbial components (2–4). Collectively, the responses produced through engagement of PRRs contribute to inflammation and constitute an important component of host defense against a large number of pathogens (5, 6). Many pathogens have devised ways to counter these protective responses by interfering with intracellular signaling events transduced through PRRs. This interference is achieved either through engagement of inhibitory receptors at the membrane or through intracellular delivery of inhibitory molecules (7–9).

Vi is a linear polymer of 1,4(2-deoxy)-2-N-acetylgalacturonic acid variably O-acetylated at the C3 position (10–12). It constitutes a major distinction between Salmonella typhi, which produces typhoid almost exclusively in humans, and nontyphoidal serovars, such as Salmonella typhimurium. The latter causes only self-limiting gastroenteritis in humans. Vi protects S. typhi from the action of anti-O Ab and renders it resistant to phagocytosis and complement-mediated killing (13). Vi also enhances survival of S. typhi in cultured macrophages (14). Typhoid rates are significantly higher in volunteers infected with capsulated serotype Typhi strains than in those infected with passaged derivatives lacking the Vi Ag. Although noncapsulated serotype Typhi strains can still cause typhoid fever, in vivo data suggest that the loss of Vi results in considerable attenuation (15). Abs to Vi protect against S. typhi infection and Vi is currently in use as a vaccine against typhoid in humans (16–19). Recently, we have shown that this polysaccharide could downregulate early chemokine secretion from intestinal epithelial cells (IECs) during infection with S. typhi by targeting the prohibitin family of molecules. This downregulation was associated with reduced activation of ERK (7).

In the current study, we show that Vi can also inhibit TLR-5-induced inflammatory responses from mononuclear phagocytes under serum-free conditions by targeting membrane-associated prohibitin and BAP-37. Serum completely abrogated these anti-inflammatory effects and converted Vi into a TLR-2 agonist. The molecule in serum that suppressed the immune-inhibitory capability of Vi and produced a proinflammatory species from it was found to be hemoglobin. These results assign a novel role to hemoglobin in thwarting anti-immune capability of typhoid virulence polysaccharide and bringing about its switch to a proinflammatory state.

Materials and Methods

Cells and reagents

The human monocytic cell lines THP-1 and U937 were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FCS at 37˚C in a humidified CO2 (5%) incubator. The LPS-hyporesponsive bone marrow-derived mouse macrophage cell line, GG2EE, was provided by Dr. Steven B. Mizel (Wake Forest University, Winston-Salem, NC). HEK-293T was maintained in DMEM supplemented with 10% FCS (DMEM-10). Vi used in this study is S. typhi-derived commercially available Vi vaccine obtained from Bharat Biotech International (Hyderabad, India). O-acetyl derivative of polylacturonic acid was prepared as described by Szewczyk and Taylor (10). Monoclonal anti-TLR-2 Ab (T2.5) was obtained from eBioscience (San Diego, CA). Human hemoglobin was purchased from Sigma-Aldrich (St. Louis, MO), and anti-Hb Abs were obtained from International (Hyderabad, India).

Abbreviations used in this paper: Apo-AI, apolipoprotein A-I; BMDC, bone marrow-derived dendritic cell; C, control; Fla, flagellin; Hb, hemoglobin; Hb-α, hemoglobin α-chain; Hb-β, hemoglobin β-chain; IB, immunoblot; IEC, intestinal epithelial cell; NC, nitrocellulose; NLR, nucleotide binding and oligomerization domain-like receptor; PRR, pattern recognition receptor; siRNA, short hairpin RNA; sRNA, small interfering RNA; SPR, surface plasmon resonance; Vi, IP from Vi-treated cells.

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obtained from Sigma-Aldrich. Abs specific to prohibitin and BAP-37 were prepared by immunizing rabbits with C-terminal peptides, which are different between prohibitin and BAP-37 (20). Animal experiments were performed according to the guidelines provided by the Institutional Animal Ethics Committee of the National Institute of Immunology.

Cell stimulation and cytokine analysis

U937 and THP-1 cells were incubated with flagellin (isolated from S. typhimurium as described by Smith et al. (21)) in the presence or absence of Vi for 6 h at 37°C in a 96-well plate in triplicate. Stimulations were performed in RPMI 1640 supplemented with or without 10% FCS. THP-1 cells were activated with Vi in the presence of serum for 6 h (for IL-8) and for 24 h (for TNF-α). For IL-6, THP-1 cells were first activated with PMA (100 ng/ml) for 24 h and then incubated with Vi for an additional 24 h in the presence of serum. Cytokines were analyzed by commercially available ELISA (Opt EIA; BD Pharmingen, San Diego, CA). Human PBMCs or immature murine bone marrow-derived dendritic cells (BMDCs) were also stimulated with flagellin in the absence or presence of Vi, and supernatants were assayed for IL-8, TNF-α, and IL-6 by ELISA.

Binding of Vi to cells and immunoprecipitation

The binding of Vi to U937 and THP-1 cells was analyzed by flow cytometry, and Vi-interacting molecules in U937 cells were identified by immunoprecipitation as described earlier (7) with slight modifications. Cells (2 × 10⁶) were washed and incubated with Vi (1 μg per 10⁶ cells) for 1 h at 4°C. Subsequently, cells were washed with PBS and lysed in TKM lysis buffer (Tris HCl 50 mM [pH 7.4], KCl 25 mM, MgCl₂ 5 mM, EDTA 1 mM, NaN₃ 0.02%, Triton X-100 1%, supplemented with a mixture of protease inhibitors). The lysate was centrifuged at 15,000 × g for 20 min before loading on protein-G-Sepharose beads preloaded with anti-Vi mAb for 4 h at 4°C. After washing with TKM lysis buffer, beads were boiled with Laemmli sample buffer (nonreducing) and run in a 12% SDS polyacrylamide gel. The immunoprecipitated proteins were transferred to an nitrocellulose (NC) membrane and blotted with rabbit anti-BAP-37 Ab, stripped, and reprobed with anti-prohibitin Ab.

Prohibitin knockdown

To obtain stable repression of prohibitin protein, 60-base-long oligonucleotides (containing a unique 19-nt sequence targeting coding regions of prohibitin) were cloned into pSUPER (neo-gfp) expression vector system (Oligoengine, Seattle, WA). The resulting transcript from recombiant vector forms short hairpin RNA (shRNA) against prohibitin. Cells were transfected using Amaxa Nuclefection Transfection kit V (Lonza, Basel, Switzerland) according to the manufacturer’s instructions. Two days after transfection, stable transfectants were selected with G418 (80 μg/ml). Knockdown of prohibitin expression was confirmed by Western blot analysis of cell lysates. The target sequences used in this study—PHB1: 5′-UGU CAA CAU CAC ACU GCG C3′, PHB2: 5′-AAT GTG GAT GCT GGG CAC AGA-3′—were chosen from previous studies (22, 23).

Primers used for RT-PCR analysis were as follows. BAP-37: Forward 5′-ATG GCC CAG AAC TTG AAG GAC T-3′; Reverse: 5′-GCT TTC TTA CCC GTT GAT GAT GCT GTC-3′; GAPDH: Forward 5′-ACC ATG GAG AAG GCT GGT-3′, Reverse 5′-CTC AGT GTA GCC CAG CAT GTC-3′.

Identification of serum proteins interacting with Vi

Vi was incubated for 48 h at 37°C with different concentrations of serum in the presence of anti-Vi Abs. The precipitates were pelleted down at 12,000 × g for 20 min at 4°C, washed gently with serum-free RPMI 1640, and run in a 12.5% SDS polyacrylamide gel, followed by silver staining. The proteins were transferred to a polyvinylidene difluoride membrane, and molecules precipitated specifically with Vi were subjected to N-terminal protein sequencing.

Surface plasmon resonance

The interaction of Vi with hemoglobin was analyzed by surface plasmon resonance (SPR) using the BIACore 2000 (GE Healthcare, Uppsala, Sweden) instrument. Purified human hemoglobin (1 μM solution in 10 mM sodium acetate buffer, pH 5.5) was immobilized on a CM-5 sensor chip using a standard amine coupling method. This coupling resulted in 2000 response units of immobilized protein on the flow cell. Binding of Vi to immobilized hemoglobin was continuously monitored in PBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20, pH 7.4). To evaluate binding, Vi was diluted in PBS buffer and analyzed at various concentrations at a flow rate of 30 μl/min in a BIACore2000. An activated and blocked flow cell without immobilized ligand was used to evaluate non-specific binding. Results were calculated using BIA evaluation version 4.1 software (BIACore). The readings obtained with nonprotein coupled flow cell were subtracted from those obtained with hemoglobin-immobilized flow cell.

Gel filtration chromatography

Vi was incubated with hemoglobin for 1 h at room temperature before subjecting it to gel filtration chromatography using BioSep-Sec-S 2000 column (Phenomenex, Torrence, CA) connected to a Shimadzu HPLC system (Shimadzu, Tokyo, Japan), with PBS as the mobile phase (flow rate, 0.25 ml/min). Alternatively, Vi alone was subjected to gel filtration chromatography. Fractions were checked for their proinflammatory activity on THP-1 cells. These fractions were also run in a 10% native polyacrylamide gel and transferred to an NC membrane. The presence of Vi in different fractions was visualized by blotting the membrane with Abs to Vi.

Transfection with TLR

HEK293T cells were transfected with various TLR-2 constructs (human TLR-2, hTLR-2/TLR-1, hTLR-2/TLR-6; Invivogen, Toulouse, France) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as the transfection reagent. Stable transfected cell lines were obtained after selection with blasticidin-S hydrochloride (10 μg/ml; Sigma-Aldrich).

Results

Vi inhibits TLR-induced cytokine responses from monocytes through interaction with the membrane-associated prohibitin complex

To analyze possible modulatory effects of Vi on inflammatory responses from monocytes, we chose the TLR-5 ligand flagellin as a model stimulus because of its major role in the induction of inflammatory responses with pathogenic Salmonella. Human monocytic cell lines (THP-1 and U937), human PBMCs, and mouse BMDCs showed reduced cytokine secretion when stimulated with flagellin in the presence of Vi (Fig. 1A–C). Treatment with Vi did not result in any detectable cell death in these cells. U937 cells incubated with Vi and subsequently stimulated with flagellin showed reduced activation of p38-MAP kinase and reduced degradation of IκBα (Fig. 1D); the former has been previously shown to be critical for TLR-5–induced IL-8 secretion from human monocytes (24). The suppression of cytokine secretion was seen even when the polysaccharide was added to cells after stimulation with flagellin (Supplemental Fig. 1), indicating that the inhibition was not due to blockade of flagellin binding to cells by Vi.

Vi showed a dose-dependent binding to human monocytes and interacted with these cells through membrane prohibitin complex (Fig. 2A, 2B). Both prohibitin and BAP-37 could be immunoprecipitated with Vi in these cells when analyzed with anti-prohibitin and BAP-37–specific Abs (Fig. 2B). The possibility that Vi might be pulling down mitochondrial prohibitin was ruled out because Vi was not internalized in these cells (up to 60 min of incubation at 37°C) plus immunoprecipitations were always performed with lysates prepared from intact viable cells incubated with Vi at 4°C.

The interaction with prohibitin complex of proteins in the membrane was essential for the anti-inflammatory capability of Vi, because knockdown of prohibitin expression using a mixture of two siRNAs targeted against exons 1 and 3 resulted in reduced binding of Vi to U937 (Fig. 2C, 2D), and consequently the inhibition in flagellin-induced IL-8 secretion mediated by Vi was also reduced (Fig. 2E). Knockdown of prohibitin expression also led to a reduction in the expression of BAP-37 protein without affecting its RNA levels (Fig. 2C, 2F), which occurs because prohibitin and BAP-37 are always present as a heterodimer and BAP-37 monomers undergo degradation in the absence of prohibitin (25). However, knockdown of prohibitin did not change constitutive expression of GAPDH, showing that siRNAs against prohibitin were target-specific (Fig.
FIGURE 1. Vi inhibits cytokine secretion from flagellin-stimulated human monocytes. A, U937 and THP-1 human monocytic cell lines were incubated with or without Vi (10 μg/ml) in serum-free medium and 1 h later stimulated with flagellin (100 ng/ml) in the absence of serum for 6 h. IL-8 was determined in the supernatants by ELISA. Data are represented as mean ± SEM. B, Human PBMCs were incubated with different concentrations of Vi in serum-free medium and 1 h later stimulated with flagellin (1 μg/ml) in the absence of serum for 24 h. IL-8, IL-6, and TNF-α were determined in the supernatants by ELISA. Data are represented as mean ± SEM. C, BMDCs were incubated with or without Vi (10 μg/ml) in serum-free medium and 1 h later stimulated with flagellin in the absence of serum for 24 h. IL-6 and TNF-α were determined in the supernatants by ELISA. D, U937 cells were incubated with or without Vi (10 μg/ml) in serum-free media for 1 h and then stimulated with flagellin (100 ng/ml) for various time points. Cell lysates were probed with Abs to phospho-p38, phospho-ERK, I-κB, and ERK. Data are representative of at least two independent experiments.

2C, 2F). These results suggest that prohibitin is required for Vi-mediated inhibition of TLR-5–induced IL-8 secretion from human monocytes.

Vi switches to a proinflammatory state in presence of serum-derived hemoglobin

The inhibition produced by Vi in flagellin-induced IL-8 secretion from monocytes was lost when cell stimulations were performed in the presence of serum (Fig. 3A), a phenomenon that was previously observed with IEC (7). The interaction with serum led to the conversion of Vi into a form that did not pull down prohibitin and BAP-37 readily (Fig. 3B). The levels of prohibitin and BAP-37 in cells incubated with Vi in the absence and presence of serum were comparable (Fig. 3B). Remarkably, the loss of inhibitory effect was associated with the switching of Vi to a proinflammatory state, because serum-transformed Vi brought about secretion of cytokines including IL-8, TNF-α, IL-6, and IL-12p40 from THP-1, human PBMCs, and mouse BMDCs (Fig. 3C–E). The stimulation of THP-1 with Vi in the presence of serum activated phosphorylation of MAPKs (p38 and ERK) and brought about degradation of I-κB (Supplemental Fig. 2). These results suggest that serum-derived factors might facilitate the interaction of Vi with an activating receptor while abrogating its binding to the inhibitory prohibitin complex.

Serum is known to upregulate LPS-mediated inflammatory responses from monocytes. This enhancement is produced through the interaction of LPS with LPS-binding protein, which transfers LPS to TLR-4-MD2-CD14 complex (26). To ensure that IL-8 secretion produced by Vi was not due to any residual LPS in the vaccine preparation used in this study, THP-1 stimulations were performed in the presence of polymyxin B. Polymyxin B abolished LPS-induced responses but did not affect Vi-induced responses (Supplemental Fig. 3A), demonstrating that the response with Vi was not due to the presence of small amounts of LPS, if any, in the Vi vaccine. Treatment with proteinase K or lipase also did not abrogate IL-8 secretion with Vi, ruling out any contaminating protein or lipid in the vaccine preparation that might contribute to inflammatory responses (Supplemental Fig. 3B, 3C). In addition, lipid extraction of Vi preparation by Bligh and Dyer’s method (27) did not result in the loss of the inflammatory response with Vi (data not shown). Importantly, depletion of Vi from the vaccine with specific anti-Vi mAb (11) preloaded on protein G-sepharose beads abrogated induction of IL-8 secretion with this polysaccharide conclusively demonstrating that the proinflammatory activity was due to Vi. There was no loss of activity when the vaccine was incubated with beads in the absence of anti-Vi Ab (Supplemental Fig. 4).

The molecule in serum responsible for transforming Vi into a proinflammatory molecule was proteinaceous in nature, because digestion with proteinase K abrogated the ability of serum to promote inflammatory responses with Vi (Supplemental Fig. 5). To identify the nature of the molecule in serum that was responsible for producing changes in Vi, an immunoprecipitation was performed by incubating this polysaccharide with FCS in the presence of anti-Vi Abs. Amino acid sequence analysis of precipitated components showed that Vi specifically interacted with hemoglobin α, apolipoprotein A-I, and fetuin (Fig. 4A; other molecules in the precipitate were also seen with anti-Vi Ab in the absence of serum and Vi), suggesting that one or more of these proteins might be involved in generating inflammatory responses with this polysaccharide and in preventing its anti-inflammatory activity. The ability to generate inflammatory responses was tested by stimulating THP-1 with Vi in the presence of hemoglobin, apolipoprotein A-I, fetuin, and BSA. The results showed that only hemoglobin could promote Vi-mediated cytokine secretion (Fig. 4B). Of the two chains of hemoglobin, β-chain was significantly more efficient than α-chain at potentiating Vi-induced IL-8 secretion from THP-1 (Fig. 4B). The interaction between Vi and hemoglobin was further established by SPR, in which Vi showed a dose-dependent binding to hemoglobin immobilized on a sensor chip (Fig. 4C). The binding with hemoglobin produced a change in the mobility of Vi in a nondenaturing gel. Being an extremely high-m.w. polymer, Vi showed poor migration into the gel. However, when it was incubated with hemoglobin or its β-chain, it migrated readily into the gel, as revealed by Western blotting with...
anti-Vi mAb (Fig. 4D), indicating a change in the physical state of Vi that likely resulted from disaggregation. This shift in the migration of Vi in the presence of hemoglobin was not readily observed with the α-chain of hemoglobin or with apolipoprotein A-I (Fig. 4D). Moreover, the mobility shift was reduced when Vi was incubated with hemoglobin in the presence of anti-Vi mAb (Supplemental Fig. 6). Furthermore, gel filtration of Vi-hemoglobin mixture demonstrated the presence of a novel Vi species that eluted later than native Vi, which normally comes out in the void volume. This faster migrating Vi, which retained reactivity with O-acetyl as well as N-acetyl recognizing anti-Vi mAbs (Fig. 4E; Vi + Hb, fractions 7 and 8), induced IL-8 secretion from THP-1 (Fig. 4F). This species was not seen when Vi alone was subjected to gel filtration chromatography (Fig. 4E; Vi). The binding of Vi to hemoglobin or its β-chain was also associated with a mobility shift in hemoglobin (Fig. 4D). Hemoglobin also mimicked the ability of serum to abrogate the inhibitory effect of Vi on the inflammatory response from flagellin-stimulated THP-1 (Fig. 4G).

**Hemoglobin-modified proinflammatory Vi engages TLR-2 to activate cellular responses**

The ability of Vi to induce secretion of inflammatory cytokines in the presence of serum was also observed with the LPS hyporesponsive murine macrophage cell line GG2EE and ex vivo peritoneal macrophages isolated from LPS hyporesponsive C3H/HeJ mice; both secreted IL-6 in response to Vi (Fig. 5A). These data suggest that TLR-4 is not involved in the induction of cytokines by Vi. This finding, combined with previous studies implicating TLR-2 in the recognition of polysaccharides (28, 29), prompted us to analyze the role of TLR-2 in inflammatory responses produced by Vi. The secretion of IL-8 from THP-1 stimulated with Vi was specifically blocked by anti-TLR-2 mAb (Fig. 5B); an isotype matched Ab did not inhibit this response. These results suggested that Vi might engage TLR-2 on THP-1 cells to generate inflammatory responses. The role of this TLR in the activation of cellular responses by Vi was confirmed by the ability of this polysaccharide to induce IL-8 secretion in the presence of serum or hemoglobin from HEK293-T cells transfected with TLR-2/TLR-1 (Fig. 5C, 5D). Non-transfected HEK293-T did not produce any IL-8 with Vi in the presence or absence of serum. Furthermore, HEK293-T transfected with TLR-2/TLR-6 responded poorly, compared with TLR-2/TLR-1, and only at a higher concentration of Vi in the presence of serum (Fig. 5C). The induction of some amount of IL-8 from TLR-2/TLR-1–transfected HEK293-T with Vi in the absence of serum might be due to higher sensitivity of these cells compared with THP-1, because these cells also responded better to the known TLR-2 agonist Pam3CSK data not shown). Serum-independent response with these transfectants also suggested that the vaccine preparation might have small amounts of pre-existing proinflammatory Vi present in it. Importantly, THP-1 and HEK293-T do not express any detectable levels of hemoglobin receptor CD163 (30, 31). Therefore, hemoglobin did not directly contribute to any intracellular signaling event during Vi-induced inflammatory responses; its action was primarily directed at the modification of Vi.

**Acetyl groups are required for the induction of inflammatory responses with Vi**

Acetyl groups have been shown previously to be important for generating Ab response against Vi (18). To study the role of these functional groups in the induction of inflammatory responses, Vi was either partially deacetylated to remove acetics from N-acetyl groups (NDeVi) or fully deacetylated to remove acetics from N-as well as O-acetyl groups (DeVi). N- and O-deacetylation was monitored by reactivity with anti-Vi mAbs recognizing different determinants on Vi (11) (Supplemental Fig. 7). IL-8 secretion from THP-1 was not observed with DeVi, suggesting that acetyl groups were required for the induction of inflammatory responses with this polysaccharide (Fig. 6A). Significantly, partially deacetylated Vi (NDeVi), which retained most of the O-acetyl groups (Supplemental Fig. 7), triggered IL-8 secretion from macrophages (Fig. 6A), indicating that O-acetics might be the critical functional determinants involved in the induction of chemokine secretion by Vi. Consistent with these results, commercially available polygalacturonic acid, which is chemically similar to Vi except for the

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**FIGURE 2.** Prohibition is required for inhibition of TLR-5-induced IL-8 secretion from human monocytes by Vi. A, Cells were incubated with different concentrations of Vi followed by anti-Vi mAb. Subsequently, cells were stained with PE-conjugated anti-mouse Ig Ab and analyzed by flow cytometry. Shaded histogram shows staining with control cells incubated with anti-Vi mAb and PE-labeled anti-mouse Ig Ab. B, U937 cells were incubated with Vi (10 µg/ml) in PBS at 4°C for 1 h. Cell lysates were immunoprecipitated with anti-Vi mAb preloaded on protein-G-sepharose beads and immunoblotted with anti-BAP-37 Ab followed by anti-prohibitin Ab. *Residual reactivity of BAP-37 Ab. C, U937 cells were transfected with empty (vector control) or prohibitin shRNA (shPhb) expression vectors. Cell lysates were blotted with Abs to prohibitin (Phb), BAP-37, and GAPDH. D, Cells were incubated with different concentrations of Vi followed by anti-Vi mAb. Subsequently, cells were stained with PE-conjugated anti-mouse Ig Ab for 1 h and analyzed by flow cytometry. Numbers on the histograms represent mean fluorescence intensities. Shaded histogram shows staining with control cells incubated with anti-Vi mAb and PE-labeled anti-mouse Ig Ab. *Residual reactivity of BAP-37 Ab. E, Control and prohibitin knockdown U937 (shPhb) cells were incubated with Vi (5 µg/ml) in serum-free medium and 1 h later stimulated with flagellin (1 µg/ml) in the absence of serum for 24 h. IL-8 was determined in the supernatants by ELISA, and percent inhibition in flagellin-induced IL-8 by Vi was calculated for each set. Data are representative of at least two independent experiments. F, Total RNA was prepared from U937 cells transfected with an empty vector or prohibitin shRNA expression vector. The relative amounts of endogenous mRNA for BAP-37 and GAPDH were analyzed by quantitative reverse-transcription PCR using gene-specific primers. C, IP from untreated cells; Vi, IP from Vi-treated cells.
lack of acetyl groups, also did not trigger any IL-8 secretion from THP-1 cells (Fig. 6A). However, O-acetylation at C-2 was not sufficient to produce inflammatory responses, because O-acetylated polygalacturonic acid also did not activate any IL-8 secretion (Fig. 6A). Therefore, in addition to the presence of O-acetyl, a specific configuration of Vi that might be produced following its interaction with hemoglobin is likely required to impart proinflammatory character to this polysaccharide. Importantly, further analysis revealed that deacetylation of Vi was associated with its inability to interact with hemoglobin. DeVi did not cause a mobility shift in hemoglobin, nor did it bind to hemoglobin bound to a chip (Fig. 6B, 6C). Alternatively, NDeVi showed a dose-dependent binding to hemoglobin (Fig. 6B, 6C).

Vi induces inflammatory responses in vivo
To establish the proinflammatory character of Vi in vivo, cytokines were analyzed in mice (C57BL/6J) injected with Vi. IL-6 was detected in the sera, and IL-6 and IL-12p40 were detected in peritoneal exudates (Fig. 6D) of mice injected with Vi in RPMI; Vi + FCS, IP from cells incubated with Vi in RPMI-1% FCS; *residual reactivity of BAP-37 Ab. C, THP-1 cells were incubated with different concentrations of Vi in the presence of serum. Supernatants were collected after 6 h for IL-8 analysis and after 24 h for TNF-α analysis. For IL-6, PMA-pretreated THP-1 cells were incubated with different concentrations of Vi in the presence of serum for 24 h. Data are represented as mean ± SEM. D, Human PBMCs from two different donors were incubated with different concentrations of Vi in the absence or presence of serum for 24 h. IL-8, TNF-α, and IL-6 were determined in the supernatants by ELISA. Data are represented as mean ± SEM. Data are representative of at least three independent experiments. E, Mouse BMDCs were incubated with Vi in the absence or presence of serum for 24 h. IL-6 and IL-12p40 were determined in the supernatants by ELISA.

Discussion
The immune system recognizes microbes through a number of membrane-associated and cytosolic sensors, including TLRs and NLRs. This sensing results in the induction of host responses that constitute key components of innate immunity and play a critical role in determining the magnitude and quality of T cell responses that provide long term immunity against pathogens. These responses are regulated through a variety of mechanisms, and many pathogens have devised ways to evade these responses, thereby promoting establishment of infection. The host-pathogen interactions involved in the induction of immune responses and their regulation during infection with S. typhi remain poorly understood because a suitable animal model for this pathogen is not available. Most of our current understanding of typhoid pathogenesis is based on studies performed in mice with S. typhimurium, which produces an infection that is analogous to human typhoid. However, considering that S. typhimurium does not produce typhoid in humans, the conclusions about human typhoid based on this mouse model need to be interpreted cautiously.

We reported previously that Vi capsular polysaccharide, which is expressed in S. typhi but not in S. typhimurium, can target the prohibitin family of molecules in IECs and bring down inflammatory responses during infection of these cells with S. typhi.
We also suggested that such a downregulation early in the gut could promote establishment of infection. Given that Vi is released in abundance during in vitro growth of *S. typhi* and it has also been reported in the sera of typhoid patients (32), and taking into account conserved expression of prohibitin in all cell types including immune cells, we reasoned that interaction of Vi with membrane prohibitin complex might also modulate immune responses from these cells during systemic dissemination of *S. typhi*. The results presented in this study demonstrate that Vi can inhibit inflammatory responses from monocytes and macrophages, which are believed to be the main cell type that harbor *S. typhi* during systemic infection. This inhibition was dependent on the interaction of Vi with a membrane complex containing prohibitin and was associated with downregulation of MAPK pathways of intracellular signaling. The inhibition mediated by Vi was lost in the presence of serum, a phenomenon that was previously observed with IECs as well (7). However, what was striking with monocytes was that the loss of inhibition in the presence of serum was associated with the induction of potent inflammatory responses with Vi. The molecule in serum responsible for bringing about this switch was found to be hemoglobin. Hemoglobin transformed Vi into a proinflammatory species that was a potent...
agonist of TLR-2/TLR-1. The interaction with hemoglobin and the induction of inflammatory responses were both dependent on the presence of O-acetyl groups in Vi. The mechanism by which hemoglobin brings about the switch of Vi from an anti-inflammatory to a proinflammatory state likely involves disaggregation of Vi followed by its transfer to TLR-2, much the same way that LPS binding protein delivers LPS to TLR-4 complex or serum vitronectin delivers BLP to TLR-2 (26, 33). However, it is possible that hemoglobin could contribute to proinflammatory activity of Vi by not only bringing about its conversion into a TLR-2 agonist but also by preventing it from engaging the inhibitory prohibitin/BAP-37 complex. The requirement for TLR-2 in the induction of inflammatory responses with Vi also explains the lack of IL-8 secretion in our previous study with the human IEC line Caco-2, which was reported to be unresponsive to TLR-2 ligands (34).

The results of this study suggest that hemoglobin might play a decisive role in guarding the host against anti-immune activities of Vi. Hemoglobin is known to exist extracellularly at concentrations ranging from 115 to 155 μg/ml, and these levels could rise further after infection with Salmonella, which has been shown to cause hemolysis in vitro (3, 35–37). Hemoglobin has been previously shown to potentiate cellular responses with LPS and LTA (3, 4, 38, 39). However, a striking difference between those studies and the present one is that here hemoglobin unveiled proinflammatory effects of Vi with ex vivo cells derived from human peripheral blood in the absence and presence of serum, respectively, provide sufficient evidence for a potent modulatory ability of circulating hemoglobin in preventing anti-immune activities of this virulence polysaccharide. The ability of hemoglobin to transform Vi into an immune activator is also supported by the...
inflammatory responses (41, 42). Our findings reveal a previously unappreciated role for circulating hemoglobin in converting a key anti-inflammatory virulence factor of S. typhi into an immune activator.

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Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1. Vi inhibits flagellin induced cytokine secretion from human monocytes. U937 cells were incubated with different concentrations of flagellin in the absence of serum and 1 h later Vi (10 μg/ml) was added and cell stimulations were carried out under serum-free conditions for another 6 h. IL-8 was determined in the supernatants by ELISA.
Supplementary Figure 2. Vi activates MAP-kinase and NF-kB pathways of intracellular signaling in presence of serum. THP-1 cells were incubated with Vi (1 μg/ml) in the presence of serum for different time points and cell lysates were probed with antibodies to phospho-p38, phospho-ERK, ERK and I-κB.
Supplementary Figure 3. Stimulation in presence of Polymyxin B, or treatment with Lipase or Proteinase K does not affect pro-inflammatory cytokine induction by Vi in presence of serum. (A) THP-1 cells were incubated with different concentrations of LPS or Vi in the presence or absence of polymyxin B (10 μg/ml) for 6 h. IL-8 was determined in the supernatants by ELISA. (B) Vi (5 μg/ml) or heat killed Streptococcus pneumoniae sonicate (10 μg/ml) was incubated with Lipase (10 μg/ml) for 1 h at 37°C followed by heat denaturation of the enzyme for 1 h at 80°C. (C) Vi (5 μg/ml) or S. typhimurium flagellin (50 ng/ml) was incubated with Proteinase K (20 μg/ml) for 1 h followed by heat denaturation of the enzyme. Untreated and Lipase - or Proteinase K -treated preparations were incubated with mouse macrophage cell line GG2EE for 24 h at 37°C. Supernatants were analyzed for IL-6.
Supplementary Figure 4. Depletion of Vi with anti-Vi antibody abrogates induction of IL-8 secretion by Vi. Vi (50 μg/ml) was incubated with Protein-G-Sepharose beads or Protein-G-Sepharose beads preloaded with anti-Vi MoAb. The bead-free supernatants were incubated with THP-1 in presence of serum and IL-8 was determined after 6 h by ELISA.
Supplementary Figure 5. Serum factor promoting IL-8 secretion with Vi is proteinaceous in nature. 10% FCS was incubated with 1 mg/ml of Proteinase K followed by heat denaturation of the enzyme. THP-1 cells were incubated with Vi (1 μg/ml) in absence or presence of untreated and enzyme-treated FCS preparations (0.1%) for 6 h. IL-8 was determined in the supernatants by ELISA.
Supplementary Figure 6. Anti-Vi abrogates mobility shift in Vi and hemoglobin. Vi (1 μg) was incubated with monoclonal anti-Vi antibody (5 μg, mouse IgG) for 1h and then with Hb (1 μg), run in a native polyacrylamide gel and transferred to nitrocellulose (NC) membrane. The NC membrane was probed with monoclonal anti-Vi antibody (mouse IgG). IB - immunoblot. Empty arrowhead indicates untreated Vi and arrows indicate mobility shift in Vi. * - Reactivity of HRP anti-mouse Ig with monoclonal anti-Vi antibody.
Supplementary Figure 7. Deacetylation of Vi with NaOH. Vi was incubated with different concentrations of NaOH (M) for 30 min, followed by pH neutralization and dialysis against PBS. Deacetylation of N- and O-acetyl groups was ascertained by reactivity with anti-Vi monoclonal antibodies directed against O-acetyl (IgM-OAc) and N-acetyl (IgM-NAc) – dependent antigenic determinants in an ELISA (Qadri et al., 1990).