The Virulence Polysaccharide Vi Released by *Salmonella* Typhi Targets Membrane Prohibitin to Inhibit T-Cell Activation

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T cells are critical to immunity against pathogenic *Salmonella* including *Salmonella* Typhi which causes systemic infection, typhoid, in humans. The strategies that this pathogen employs to keep T-cell mediated immune responses in check during establishment of systemic infection are not completely understood. Here, we show that the virulence polysaccharide Vi, which distinguishes *S*. Typhi from localized gastroenteritis-producing non-typhoidal *Salmonella* serovars, is a potent inhibitor of T-cell activation. Vi released by *S*. Typhi interacts with the membrane prohibitin complex and inhibits IL-2 secretion from T cells stimulated through the T-cell receptor (TCR) but does not affect PMA-activated interleukin 2 (IL-2) secretion. Treatment with Vi suppresses early activation events including TCR down-regulation, actin polymerization, and phosphorylation of ERK. Co-administration of Vi with anti-CD3 Ab reduces secretion of IL-2 and interferon γ in mice. Our findings reveal a mechanism by which *S*. Typhi may target T-cell immunity during establishment of typhoid.

**Keywords.** *Salmonella* Typhi; virulence polysaccharide Vi; prohibitin; T cells.

*Salmonella* Typhi enters the body through the oral route, invades the intestinal epithelium, and disseminates throughout the reticuloendothelial system. It produces this systemic infection almost exclusively in humans [1, 2]. Due to this extreme host specificity, the pathogenesis of typhoid infection is poorly understood. Unlike *S*. Typhi, *Salmonella* Typhimurium produces a systemic infection in susceptible strains of mice that is analogous to human typhoid. Therefore, infection of mice with *S*. Typhimurium has been used as a model to understand pathogenesis of human typhoid [3]. Even though this model does not truly represent *S*. Typhi infection in humans as *S*. Typhimurium does not cause typhoid in humans, it has provided important insights into *Salmonella* pathogenesis in general. Studies carried out with this model have demonstrated that T cells and T-cell-derived cytokines such as interferon γ (IFN-γ) are required for clearance of bacteria and for long-term immunity against *Salmonella* [4–7]. Human volunteers immunized with vaccine strains of *S*. Typhi showed activation of CD4+ and CD8+ T cells, which had protective characteristics similar to those reported for T cells in the mouse model [8]. Collectively, these studies have suggested an important role for T cells in protective immunity against *S*. Typhi. How *S*. Typhi might thwart T-cell immunity during establishment of typhoid is unclear.

One of the major distinctions between *S*. Typhi and *S*. Typhimurium is the presence of virulence polysaccharide Vi in *S*. Typhi [9]. This polysaccharide prevents complement-mediated killing of *S*. Typhi and renders this pathogen resistant to phagocytosis [10]. We have previously shown that Vi can suppress inflammatory responses from intestinal epithelial cells and monocytes through its interaction with membrane prohibitin [11, 12]. Prohibitin belongs to a family of evolutionarily conserved proteins that include the flotillins, stomatin and the stomatin-like protein, podocin, and erlins. These proteins have been implicated in a large number of cellular functions such as protein trafficking, stabilization of mitochondrial respiratory chain complexes, cell...
proliferation, and apoptosis and cell signaling [13–15]. Prohibitin and its related members have been reported to be associated with antigen receptors in B cells [16]. More recently, prohibitin has been found to be important for raf-ras--dependent activation of extracellular regulated kinase (ERK) in epithelial cells [17].

In this study, we provide evidence that targeting of membrane prohibitin in T cells by Vi released from S. Typhi can down-regulate T-cell responses. Treatment with this polysaccharide inhibited T-cell receptor (TCR)--induced intracellular signaling including activation of MAP-kinases and suppressed cytokine secretion in vitro as well as in vivo. The expression of Vi might therefore provide S. Typhi a strategy to interfere with T-cell activation.

**RESULTS**

**Vi Suppresses Antigen Receptor–Induced Cytokine Secretion From T cells**

To study possible effects of Vi on T-cell activation, we first analyzed binding of Vi to T cells. Vi showed a dose-dependent binding to the model human T-cell line Jurkat (Figure 1A). This binding was also observed with bacterial culture supernatant obtained from Vi positive but not Vi negative S. Typhi indicating release of this polysaccharide during growth of bacteria in vitro (Figure 1B). Significantly, the release of Vi and its interaction with T cells was also seen during infection of splenocytes with Vi positive S. Typhi ex vivo (Figure 1C). Vi readily bound to peritoneal T cells in vivo (Figure 1D).

To investigate if binding of Vi to T cells could modulate T-cell response, Jurkat cells were stimulated through the T-cell receptor in the presence or absence of this polysaccharide, and interleukin 2 (IL-2) was analyzed in cell supernatants. Because our previous study had shown that the anti-inflammatory effect of Vi on human monocytes could be modulated by serum and serum-derived hemoglobin (Hb) [12], we carried out stimulations under serum-free and serum-supplemented conditions to see if that phenomenon existed in T cells as well. Vi inhibited IL-2 secretion from Jurkat in a dose-dependent manner in the absence of serum (Figure 2A). This inhibition was not due to blockade of binding of anti-TCR antibody to cells or induction of cell death (Figure 2B and 2C). Vi did not inhibit PMA/ionophore - activated IL-2 secretion from Jurkat (Figure 2D). The lack of suppression with Vi in presence of serum or serum-derived hemoglobin reaffirmed the role of circulating Hb in neutralizing immune-inhibitory capability of Vi (Figure 2E and 2F). The inhibition brought about by Vi was not restricted to Jurkat as the polysaccharide also inhibited cytokine secretion from peripheral blood-derived human lymphocytes activated ex vivo with anti-CD3 antibody plus anti-CD28 antibody, as well as from mouse T cells activated with anti-TCR antibody or specific peptide-MHC complexes (Figure 2G–I). Vi-mediated inhibition in IL-2 secretion from activated T cells was also mimicked by bacterial culture supernatant derived from Vi positive S. Typhi but not by supernatants obtained from Vi-deficient S. Typhi or other bacterial strains (Figure 2J). The down-regulation of IL-2 secretion required presence of acetyl groups in the polysaccharide as this effect was not observed with deacytlated Vi (Figure 2K and 2L). It was also not seen with the capsular polysaccharide derived from *Streptococcus pneumoniae* pneumococcal capsular polysaccharide (PCP) or lipopolysaccharide (LPS) isolated from *Salmonella* (Figure 2K and 2L), suggesting that the ability to suppress IL-2 secretion from antigen receptor activated T cells was specific to Vi.

**Vi Engages Membrane Prohibitin Complex and Inhibits Early Activation Events**

To get insights into how Vi inhibits T-cell activation, further experiments were carried out with Jurkat. Immunoprecipitation with Vi revealed that the Vi-recognition complex in T cells composed of prohibitin associated with its closely related homolog BAP-37, similar to what we had previously reported with IECs and monocytes (Figure 3A) [11, 12]. Microscopic analysis showed punctuate staining with Vi, indicating that the membrane prohibitin complex interacting with this polysaccharide might be localized within specific membrane domains (Figure 3B). Similar staining pattern was observed during interaction of T cells with Vi+ S. Typhi but not with Vi- S. Typhi or S. Typhimurium (Figure 3B).

To analyze effect of Vi-prohibitin interaction on early activation events, we looked at TCR down-regulation from the surface following activation with anti-CD3 antibody or PMA. Vi suppressed internalization of TCR in cells activated with anti-CD3 antibody without affecting PMA-activated TCR internalization (Figure 3C and 3D). In fact, PMA-induced TCR internalization was increased in presence of Vi (Figure 3). Vi also inhibited anti-CD3 Ab-induced CD69 expression and ERK phosphorylation in Jurkat (Figure 3E and 3F), but it did affect PMA-activated ERK phosphorylation (Figure 3F).

The activation of TCR-triggered proximal signaling events is largely regulated through actin cytoskeletal rearrangements [18]. We therefore considered the possibility that ligation of membrane prohibitin complex with Vi might interfere with actin polymerization. This was investigated by analyzing staining of cells with the fungal toxin Phalloidin, which binds filamentous actin. A significant proportion of cells treated with Vi for 1 hour at 37°C showed reduced binding to Phalloidin indicating depolymerisation of preexisting actin filaments (Figure 4). This effect was time-dependent, and recovery of actin polymerization was observed at later time point (Figure 4). These results suggested that ligation of membrane prohibitin with Vi during TCR activation might interfere with intracellular signaling through modulation of actin cytoskeletal rearrangements in Jurkat cells.
Vi Suppresses T-Cell Activation in vivo

The effect of Vi on T-cell activation in vivo was examined by analyzing secretion of cytokines upon administration of anti-CD3 antibody. Our previous study had shown that depending on how Vi was presented to mononuclear phagocytes, Vi could either be anti-inflammatory or generate inflammatory responses [12]. Therefore, to circumvent any interference from such inflammatory responses on the ability of Vi to modulate T-cell activation, we examined the effect of Vi on T-cell activation in vivo.

**Vi binds T cells.** Jurkat cells were incubated with different concentrations of Vi (A) or with *Salmonella* Typhi–derived bacterial culture supernatants (B) for 1 hour at 4°C followed by anti-Vi MoAb and FITC-labeled anti-mouse Ig Ab. Cells were analyzed by flow cytometry. Control cells (shaded histogram) were incubated only with anti-Vi MoAb and FITC-anti-mouse Ig Ab. Data were plotted using WinMDI software. C.S., culture supernatant. C, Splenocytes isolated from C57Bl/6 mice were infected with *S*. Typhi at different MOI. One hour later, T cells were stained with PE-labeled anti-TCR antibody and Vi followed by anti-Vi MoAb and FITC-labeled anti-mouse Ig Ab. Cells were analyzed by flow cytometry. The results are representative of 2–3 independent experiments. D, Mice were injected with 100 µg Vi or equivalent volume of PBS intraperitoneally. One hour later, cells isolated from the peritoneum were incubated with anti-Vi MoAb and FITC-labeled anti-mouse IgG followed by PE-labeled anti-TCR antibody for 1 hour. Cells were run in BD Accuri flow cytometer, and data were analyzed using FlowJo. The results are representative of 2 independent experiments of 2 mice each. Abbreviations: Ab, antibody; IgG, immunoglobulin G; MOI, multiplicity of infection; PBS, phosphate-buffered saline; TCR, T-cell receptor; Vi, virulence polysaccharide.
responses in vivo, we employed MyD88 deficient mice, which do not produce cytokine responses with Vi (authors’ unpublished data). Mice were injected with anti-CD3 antibody in the absence or presence of Vi, and IL-2 and IFN-γ levels were determined in sera. Mice injected with anti-CD3 antibody readily produced IL-2 and IFN-γ (Figure 5A and 5B). However, this response was significantly reduced in mice, which received Vi along with anti-CD3 antibody (Figure 5A and 5B).

**DISCUSSION**

S. Typhi causes systemic infection typhoid in humans, whereas nontyphoidal *Salmonella* serovars such as *S. Typhimurium* produce only self-limiting gastroenteritis that is associated with a potent inflammatory response in the gut [19]. To date, very little is known about the identity of specific host-pathogen interactions that might be responsible for different clinical manifestations.
produced by these closely related Salmonella serovars in humans. Clearly, S. Typhi must be modulating immune activities in a manner that allows it to establish systemic infection. How these modulations are brought about is not completely understood. A major difference between S. Typhi and S. Typhimurium is the presence of Vi capsular polysaccharide in the former. Vi protects...
Figure 3. Vi interacts with membrane-associated prohibitin family of molecules and suppresses anti-CD3 antibody-induced but not PMA-induced early activation events. A, Jurkat cells (3 × 10⁷ cells) were incubated with Vi (1 µg/10⁶ cells) in RPMI-1640 for 1 hour at 4°C. Cells were washed, lysed with TKM buffer (Tris. HCl, 50 mM, pH7.4, KCl 25 mM, MgCl₂ 5 mM, EDTA 1 mM, NaN₃ 0.02%, Triton X-100 1%, supplemented with a cocktail of protease inhibitors), and lysates were immunoprecipitated with anti-Vi Ab loaded on Protein G-Sepharose beads. The beads were washed, boiled with Laemmli sample buffer, and electrophoresed in a 12% SDS-PAG. Proteins were transferred to a nitrocellulose sheet and probed with prohibitin and BAP-37 specific rabbit Ab. The blot was developed using ECL. Lane 1: cell lysate, lane 2: immunoprecipitation with cell lysate from untreated cells, lane 3: immunoprecipitation with lysate from Vi-treated cells. B, Cells incubated with Vi (1 µg/mL) at 4°C or infected with Vi⁺ S. Typhi, Vi⁻ S. Typhi or S. Typhimurium, followed by incubation with anti-Vi antibody and Alexa fluor-594-labeled antimouse Ig were analyzed by confocal microscopy. C and D, Jurkat cells were activated with plate-coated anti-CD3 antibody for 6 hours and incubated with APC-labeled anti-CD69 antibody for 1 hour at 4°C. Cells were analyzed in a flow cytometer. Shaded histogram represents staining with FITC-labeled anti-mouse IgG alone. E, Jurkat cells were activated with plate-coated anti-CD3 Ab for 6 hours and incubated with APC-labeled anti-CD69 antibody for 1 hour at 4°C. Cells were analyzed in a flow cytometer.
S. Typhi from complement-mediated killing and phagocytosis [9, 10]. We have previously reported that this polysaccharide can interact with membrane associated prohibitin family of molecules in IECs and monocytes and bring down inflammatory responses [11, 12]. The results presented here show that S. Typhi releases considerable amounts of Vi during in vitro infection, which can readily bind T cells through prohibitin and specifically inhibit antigen receptor driven T-cell activation. These results suggest that Vi can impair T-cell mediated immune responses during typhoid even in the absence of a direct contact between the pathogen and T cells. This impairment would be very effective at anatomical sites which express low levels of circulating hemoglobin as the latter prevents interaction of Vi with membrane prohibitin and abolishes its inhibitory capacity [[12]; present study]. Therefore, gut, which unlike spleen does not harbor large numbers of erythrocytes, could be a prime site where Vi would be able to exert its effect maximally. Vi-mediated inhibition in the gut may also be increased due to higher levels of prohibitin at this site (authors’ unpublished data). Considering that gut is the site where S. Typhi infection is initiated, the released polysaccharide would empower S. Typhi to prevent T-cell activation right at the beginning of infection. Vi has been previously reported in circulation in typhoid patients [20,21]. Kullas et al [22] have recently reported that S. Typhimurium produces asparaginase, which can also down-regulate T-cell responses. However, the expression of this molecule and its possible role during S. Typhi infection has not been tested.

The exact mechanism by which interaction of Vi with membrane prohibitin inhibits T-cell responses is not clear at the moment. Similar to IECs and monocytes, the Vi-recognition
complex in T cells was found to comprise of membrane prohib-
itin and BAP-37 [11, 12]. Prohibitin has been previously shown
to regulate ras-raf–dependent activation of ERK in epithelial
cells [17]; therefore, it is possible that engagement of membrane
prohibitin complex with Vi modulates ERK phosphorylation in
T cells through a similar mechanism. Signifi-
cantly, engagement of membrane prohibitin with Vi promoted actin
depolymerisation, which could abrogate activation of a large repertoire of signaling
intermediates including MAP-kinases during TCR signaling as actin polymerization is vital to generation of signaling clusters
during T-cell activation [18, 23]. We have data to suggest that membrane prohibitin complex might regulate activation of
the src kinase Lck (authors’ unpublished data); the latter has
a central role in TCR signaling [24]. The effect on Lck activa-

tion, which could well be through modulation of actin
cytoskeletal rearrangements by Vi, might explain why Vi inhib-
its anti-TCR Ab–mediated TCR internalization without affect-
ing PMA-activated TCR internalization as the former is
mediated primarily through tyrosine phosphorylation depen-
dent events, whereas the latter is produced through serine phos-
phorylation [25]. Similar dichotomy has been previously
reported during inhibition of human T-cell activation with choler toxin [26]. The reasons for differential modulation of
tyrosine vs serine phosphorylation by the membrane prohibitin complex are currently under investigation in our laboratory.

In conclusion, our findings have revealed a very efficient
mechanism by which Salmonella Typhi could target the T-cell
arm of the immune system at hemoglobin-low locations, nota-

gly the gut that is the primary site of Salmonella infection, and
thus promote establishment of its systemic infection.

**MATERIALS AND METHODS**

**Mice**
MyD88 knockout mice on C57BL/6 background were obtained
from Jackson Laboratories, United States, and maintained in the
small animal facility of the National Institute of Immunology.
Mice were maintained in germ-free conditions. Animal experi-
ments were carried out according to the guidelines provided by
the Institutional Animal Ethics Committee.

**Cell Lines**
The human leukemic T-cell line Jurkat was obtained from ATCC, Manassas, VA. Myelin basic protein (MBP)-specific mu-
rine T-cell hybridoma 1934.4 was provided by Prof Sally Ward,
UT, Southwestern Medical Center, Dallas, TX. Mononuclear
cells were isolated from human peripheral blood mononuclear
cells (PBMC) by Ficoll-Hypaque density centrifugation; nonad-
herent PBMC were used for activation purposes. Blood was col-
lected according to the guidelines outlined by the Institutional Human Ethics Committee.

**Antibodies and Other Reagents**
Polyclonal rabbit antibodies were generated against prohibitin
and BAP-37 as described previously by Coates et al, 1997 [27]. All other antibodies used in this study were from Millipore
or Cell Signaling, United States. Vi was obtained from Aventis
Pasteur Connaught, India, or Bharat Biotech Limited, India,
and dialyzed against phosphate-buffered saline (PBS) before
using in cellular studies. The de-acetylated derivative of Vi
was prepared as described by Szewczyl and Taylor, 1980 [28].
S. Typhosa LPS was from Sigma Chemical Co, United States.
Capsular polysaccharide isolated from Streptococcus pneu-
moniae (PCP) was kindly provided by Dr Devinder Sehgal, NIH.
Alexafluor 488-labeled Phalloidin was from Molecular Probes
and FITC-labeled anti-mouse Ig antibody was from Jackson
Laboratories, United States.
Activation of T cells
To investigate the effect of Vi on IL-2 secretion, T cells were activated with anti-CD3 Ab (10 µg/mL) plus anti-CD28 Ab (5 µg/mL) in the absence or presence of Vi, DeVi, PCP, or LPS (Jurkat & mouse T-cell hybridoma for 18–20 hours and ex vivo human T cells for 48 hours) at 37°C after which the supernatants were analyzed for IL-2 by enzyme-linked immunosorbent assay (ELISA). Murine T-cell hybridoma was also activated with a specific peptide along with antigen presenting cells. Splenocytes isolated from C57Bl/6 mice were activated with anti-CD3 antibody for 48 hours. In some experiments, Jurkat cells were activated with PMA (100 ng/mL) and ionophore (100 ng/mL), and IL-2 was determined in the supernatants after 8 hours. Cell death was examined by propidium iodide uptake using flow cytometry.

To analyze effect of Vi on TCR down-regulation, T cells were activated with plate coated anti-CD3 antibody or PMA in the absence or presence of Vi for 1 hour, washed and incubated with anti-CD3 antibody, followed by PE-labeled anti-mouse Ig antibody. Subsequently, cells were washed and analyzed in a flow cytometer (BD, LSR). Data were plotted using WinMDI.

The effect of Vi on TCR-induced expression of CD69 in Jurkat was also investigated by flow cytometry. Cells were analyzed in BD, Accuri, and the data were plotted using FlowJo.

Binding of Vi to Cells and Identification of Vi-recognition Complex
The binding of Vi to cells and identification of Vi-binding molecules was carried out as described elsewhere [11].

Confocal Microscopy
Cells were incubated with Vi (1 µg/mL) for 1 hour at 4°C followed by anti-Vi MoAb and Alexafluor-594 - conjugated anti-mouse Ig Ab. Alternatively, cells were infected with Vi+ S. Typhi, Vi- S. Typhi, or S. Typhimurium at a MOI 50, washed and incubated with anti-Vi MoAb, followed by Alexafluor-594 labeled anti-mouse Ig antibody. Images were acquired in a confocal microscope (Olympus FluoView FV1000) and transferred to Adobe Photoshop for printing. Control cells were incubated only with anti-Vi MoAb and Alexafluor-594-conjugated anti-mouse Ig Ab.

Analysis of Actin Polymerization
Cells were incubated with PBS or Vi (5 µg/mL) at 37°C in serum-free RPMI-1640 for 1 hour or 5 hours at 37°C. Cells were washed, fixed with 4% paraformaldehyde for 20 minutes at room temperature, and stained with Alexafluor 488-labeled phalloidin. Cells were analyzed in a flow cytometer.

Effect of Vi in vivo
MyD88 deficient C57Bl/6 mice (3–4 per group) were injected intraperitoneally with Vi (100 µg/mouse) or the vehicle (PBS) followed half an hour later with anti-CD3 antibody (50 µg/mouse). Mice were bled prior to and 3 hours after injecting the antibody and cytokine levels were determined in the sera by ELISA. All animal experiments were carried out according to the guidelines provided by the Institutional Animal Ethics Committee of the National Institute of Immunology.

Statistical Analysis
Values are expressed as mean±SD. Statistical analysis was performed using paired Student t-test except for the in vivo experiment in which unpaired Student t-test was employed. A P value of <.05 was considered statistically significant in all analyses.

Notes
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References